The Dissertation Committee for Sachin Thakkar Certifies that this is the approved version of the following dissertation:

Novel dry powder formulations of aluminum salt-adjuvanted vaccines for intranasal administration

Committee:

Zhengrong Cui, Supervisor

Robert O. Williams III

Hugh D. Smyth

Christopher A. Jolly

Christopher R. Frei

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by

Sachin Thakkar

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Dedication

I would like to dedicate this work to Lord Swaminarayan and my spiritual master Guruhari Hariprasad Swamiji. I would also like to dedicate this work to Pujya Prabodhjivan Swamiji, Bhaktipriya Swamiji, Kishanji, Parth Bhai, and to my dear Atmiya friends. Finally, this work is also dedicated to my parents Girishchandra Thakkar, Shobhanaben Thakkar, sister Hetal Thakkar, and my Jalpa Thakkar who have worked hard all their life for making sure I can achieve all my dreams.

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Novel dry powder formulations of aluminum salt-adjuvanted vaccines for intranasal administration

Sachin Thakkar, Ph.D.

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Supervisor: Zhengrong Cui

Abstract: Many currently licensed and commercially available human vaccines contain insoluble aluminum salts as vaccine adjuvants. The primary particles of aluminum (oxy)hydroxide and aluminum (hydroxy)phosphate are in the nanometer-scale. However, when dispersed in an aqueous solution, the primary particles aggregate to form larger microparticles of 1-20 μm. A major limitation with these vaccines is that they must not be exposed to freezing temperatures during transport or storage such that the liquid vaccine freezes, because slow freezing causes irreversible coagulation that damages the vaccines (e.g., loss of efficacy). Therefore, vaccines that contain aluminum salts as adjuvants are formulated as liquid suspensions and are required to be kept in a cold chain (2-8^{o} C) during transport and storage. Formulating vaccines adjuvanted with aluminum salts into dry powder that can be readily reconstituted before injection could address this limitation. Spray freeze-drying of vaccines with low concentrations of aluminum salts and high concentrations of trehalose alone, or a mixture of sugars and amino acids, as excipients can convert vaccines containing aluminum salts into dry powder, but fails to preserve the

particle size and/or immunogenicity of the vaccines. In this dissertation, using ovalbumin as a model antigen adsorbed onto aluminum (oxy)hydroxide or aluminum (hydroxy)phosphate, a commercially available tetanus toxoid vaccine adjuvanted with potassium aluminum sulfate, a human hepatitis B vaccine adjuvanted with aluminum (oxy)hydroxide, and a human papillomavirus vaccine adjuvanted with aluminum hydroxyphosphate sulfate, it was showed that vaccines containing a relatively high concentration of aluminum salts (up to ~1%, w/v, of aluminum hydroxide) can be converted into a dry powder by thin-film freeze-drying by using low levels of trehalose (i.e., as low as 2% w/v) as an excipient. Importantly, the thin-film freeze-drying process did not cause particle aggregation, nor decreased the immunogenicity of the vaccines upon reconstitution. Moreover, we showed that the immunogenicity of thin-film freeze-dried OVA-adsorbed Alhydrogel® vaccine powder was not significantly changed after it was exposed for an extended period of time in temperatures as high as 40° C or subjected to repeated slow freezing-and-thawing. Nasal vaccination using a dry powder vaccine formulation represents an attractive, non-invasive delivery with better storage stability and provides added protection at mucosal surfaces. In this dissertation, we also demonstrate the feasibility of nasal immunization using the dry powder formulation of insoluble aluminum salt adjuvanted vaccines and a novel intranasal (IN) delivery technology. Special emphasis was put on the characterization of the formulation that can be realistically used in humans by a nasal dry powder delivery device. The dry powder vaccine elicited a significant serum antibody response in rats compared to that of liquid vaccine administered via subcutaneous injection or IN route. Significant mucosal specific IgA responses were also observed solely

after IN delivery. In addition, in vitro nasal deposition study using nasal casts of adult humans using a novel nasal dry powder device shows that out of the total recovered powder developed showed that out of the recovered powder (~60%), about 90% stayed in the nose. This study demonstrates for the first time to our best knowledge the generation of potent systemic and mucosal immune responses using the dry powder form of an aluminum salt-adjuvanted vaccine and suggests dry powder vaccine formulations are a promising approach for mucosal vaccination targeting the nasal mucosa. Thin-film freeze-drying is a viable platform technology to produce dry powders of vaccines that contain aluminum salts. It is expected that immunization programs can potentially benefit by integrating thin-film freeze-drying into vaccine preparations.

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Chapter One

Introduction

1.1 INTRODUCTION TO VACCINE ADJUVANTS

Immunization is one of the most powerful and cost-effective of all health interventions (1). Immunization averts an estimated 2.5 million deaths a year. For example, with the help of immunization, polio has been eradicated in three of World Health Organization's (WHO) six regions and is today endemic in only four countries, down from 125 countries in 1988. A vaccine formulation generally includes various components. One major component of a vaccine formulation is the antigen. An antigen is any molecule that can bind specifically to an antibody and has the potential to generate an immune response (2). In the conventional vaccines, the antigen usually consists of dead bacteria, dead or live attenuated virus, or toxoid. These antigens are highly immunogenic, but they can potentially cause human health hazards as well. The newer generation vaccines consist of recombinant proteins, peptides, or polysaccharides as an antigen. The newer generation vaccines are preferred for safety reasons, but they are only weak immunogenic and usually require additional components to help stimulate strong and protective immunity (2). These components are termed as vaccine 'adjuvants'. An adjuvant is any substance that can enhance the immune responses to an antigen with which it is mixed, but is not immunogenic itself (3, 4). A vaccine adjuvant may enhance antigen-specific immune responses, prolong immune responses, and/or affect the type of immune responses. In

addition, use of a vaccine adjuvant may also reduce the number of immunization required in an immunization protocol and help to develop single dose immunization that can reduce the overall immunization costs. Ramon (1925) first demonstrated that it is possible to increase levels of diphtheria or tetanus antitoxins by the addition of bread crumbs, agar, tapioca, starch oil, lecithin, or saponin to diphtheria or tetanus vaccines (5).

1.2 ALUMINUM SALTS AS VACCINE ADJUVANT

Mineral salts such as insoluble aluminum salts and calcium phosphate have been used as adjuvants in vaccine formulations. Aluminum salt-based adjuvants have been used in immunization programs for decades to help induce early, high-titer, and long-lasting protective immunity. Aluminum compounds, including aluminum (hydroxy)phosphate ($AlPO_4$), aluminum (oxy)hydroxide ($Al(OH)_3$), and alum precipitated vaccines - historically referred to as protein aluminate - are currently the most commonly used adjuvants in human and veterinary vaccines. According to the WHO, in 2015, the global vaccine market is valued more than $41 billion, compared to $24 billion in 2013. Out of which, aluminum salt-adjuvanted vaccines take up more than 50% of them (6-8). Diphtheria-tetanus-pertussis vaccines, hepatitis A vaccines, hepatitis B vaccines, pneumococcal conjugate vaccines, anthrax vaccine, and rabies vaccines, all contain aluminum salts as adjuvants (9-12). In fact, 9 out of the top 15 best-selling vaccines contain an aluminum salt as an adjuvant (8, 9).

Aluminum salt-containing adjuvants are often referred to as ‘alum’ in the literature, which is deceptive, because (1) alum, chemically potassium aluminum sulfate

($KAl(SO_4)_2 \cdot 12H_2O$), has not been widely used as an adjuvant in human vaccines, and (2) aluminum hydroxide and aluminum phosphate have different physical characteristics (13) and differ in their adjuvant properties (14). Potassium alum was originally used to partially purify protein antigens, particularly tetanus and diphtheria toxoids, by precipitating them in the presence of anions including phosphate, sulfate, and bicarbonate, resulting in a mixture of compounds, mainly aluminum phosphate and aluminum hydroxide (15, 16). While aluminum hydroxide and aluminum phosphate adjuvants are chemically referred to as $Al(OH)_3$ and $AlPO_4$, they are not stoichiometrically true. Aluminum hydroxide is a crystalline aluminum oxyhydroxide, which is positively charged at physiological pH (i.e., isoelectric point (pI) = 11) (14, 17, 18). Aluminum phosphate is an amorphous aluminum (hydroxy)phosphate, which is negatively charged at physiological pH (pI = 5–7) (14, 17, 18). One of the earliest uses of an aluminum salt as an adjuvant was reported by A. T. Glenny and co-workers in 1926, who showed that the suspension of alum-precipitated diphtheria toxoid has a higher immunogenicity than the toxoid alone (19). The use of alum-precipitated tetanus and diphtheria toxoids has declined considerably because of the variability in production of alum-precipitated toxoids (12, 15, 16, 20). However, aluminum adjuvanted vaccines have become widely used since then. The immunogenicity of antigens adsorbed onto aluminum salt-based adjuvants depends on several factors, but the most important include the degree of adsorption of the antigens onto the adjuvants and the dose of adjuvants (11, 13, 17, 21).

The search for alternative and improved vaccine adjuvants continues today, but aluminum salt-containing adjuvants are like to remain a mainstay in vaccine formulations

due to their excellent record of safety (11-14, 16, 21, 22). Due to the popularity of the aluminum compounds as adjuvants in human vaccines, they have become the point of reference for evaluating new adjuvants. Although aluminum salts have been used for decades, their methods of preparation remain traditional, in which antigens are simply adsorbed onto adjuvants. The primary particles of aluminum hydroxide are in the nanometer-scale, however, due to their poor water solubility, when dispersed in water, the primary particles aggregate to form larger microparticles of 1-20 μm. Therefore, a vaccine that is prepared by binding an antigen with an aluminum salt is physically a suspension of aluminum salt particles with antigens adsorbed onto them. However, adsorption of antigens on aluminum salt-based adjuvants depends on various factors including physical and chemical characteristics of the antigens, the type of aluminum salt used, conditions of adsorption, charges on adjuvants and antigens, the pH of the formulation, the size of the aluminum salt particles, the order of addition of reagents, and the speed of mixing (13, 14, 21-25). Because it is difficult to manufacture aluminum adjuvants consistently and reproducibly, and a poorly formulated aluminum adjuvant preparation does not have the optimal adjuvant activity (23, 26), Alhydrogel® (aluminum (oxy)hydroxide) and Adju-phos® (aluminum (hydroxy)phosphate) from Superfos Biosector (now Brenntag Biosector, A/S) have been chosen as a scientific standard in an independent scientific workshop in Greece for the evaluation of new adjuvants and to minimize batch-to-batch variability (27, 28).

1.3 MECHANISM OF ACTION FOR ADJUVANT ACTIVITY OF ALUMINUM SALTS

Many human vaccines contain insoluble aluminum salts as adjuvants (29, 30). Although insoluble aluminum salts have been used in vaccines for decades, their exact mechanisms of action have baffled scientists for years (31, 32). Over the years, various theories have been proposed to explain the mechanisms underlying the adjuvant activity of insoluble aluminum salts. It is clear now that aluminum salts work by a mixture of different mechanisms. Proposed mechanisms of immunopotentiation by aluminum-containing adjuvants include formation of antigen depot (33, 34), stimulation of antigen-presenting cells such as dendritic cells (DCs) (35), complement activation (36), and stimulation of chemokine release (31, 36). Moreover, there are reports showing that aluminum salts activate intracellular pathogen pattern recognition receptor signaling pathway involving the NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome (37, 38). Potent inflammatory cytokines such as IL-1β and IL-18 are released in response to NLRP3 activation, which direct the host responses to infection and injury (39). Furthermore, Kool and colleagues reported that the immunopotentiating effect of aluminum salts depends on the induction of uric acid (40).

Previously we and others discovered that adjuvant activity of aluminum (oxy)hydroxide and aluminum (hydroxy)phosphate could be significantly improved by reducing the size of the particles in the aqueous suspensions of the insoluble aluminum salts from micrometer scale to nanometer scale (e.g. from 1-20 μm to ~100 nm) (41-44). We further provided evidence that the more potent adjuvant activity of the aluminum salt

nanoparticles is likely related to their stronger ability in activating the NLRP3 inflammasome than aluminum salt microparticles (42).

1.4 LIMITATIONS OF ALUMINUM SALT-ADJUVANTED VACCINES

1.4.1 Freezing of vaccines is critical issue

The size of aluminum hydroxide and aluminum phosphate particles is in the nanometer range. However, these particles when dispersed in aqueous solution aggregate to make microparticles of 1-20 μm (54). Thus, a vaccine that is prepared by binding an antigen with an aluminum salt is physically a suspension of aluminum salt particles with antigens adsorbed on them. In order to maintain highest level of potency, the vaccines must be kept in cold-chain (2-8˚C) during storage and transport (55). A major limiting factor with these vaccines is that they must not be exposed to freezing conditions during transport and storage. Inadvertently exposing the suspension to freezing temperatures causes irreversible coagulation of aluminum salts that damages the vaccines (e.g., loss in potency and stability) (55).

Despite the best efforts, temperature excursions during cold-chain is common for many reasons such as improperly maintained or outdated refrigerator, loss of power, poor compliance with cold-chain procedures, inadequate monitoring and poor understanding of the dangers of freezing the vaccines (56). Vaccines that have been accidentally exposed to freezing conditions before administration to patients must be discarded, causing significant product waste and limited utility. This is significant considering that estimated 75-100% of vaccine shipments are exposed to freezing temperatures at some time during shipment

(57). This is a budding issue as number of freeze-sensitive vaccines increases. It is estimated that freeze-sensitive vaccines represent over 31% of the US $439 million UN Children's fund spent on all vaccines (57).

Meanwhile, as the costs and/or logistical constraints of vaccine delivery associated with the cold-chain requirements significantly obstructs global vaccine access, there is increasing interest in novel approaches to vaccine stability management such as controlled temperature chain (CTC) storage (45, 46). CTC allows vaccines to be managed in temperatures outside of the traditional cold-chain for a limited period of time, typically a single excursion into ambient temperature not exceeding 40° C for the duration of a specific number of days prior to administration (47). For example, in 2012, MenAfriVac™ became the first to be prequalified by the World Health Organization (WHO) to receive regulatory approval during mass vaccination that allowed vaccine storage at or below 40° C for up to 4 days (45, 48, 49). It is estimated that by using CTC, the costs of using cold-chain and the associated logistics can be reduced by 50% (48).

1.4.2 Aluminum salts are weakly immuniogenic

It is well-known that aluminum salts as adjuvants predominantly help induce antigen-specific humoral immune responses or T helper type 2 (Th2) biased cellular immune responses (50). Aluminum salt-adjuvanted vaccines can only weakly boost specific antibody responses, and its inability to help induce cellular immune responses has triggered researchers to search for alternative adjuvants (21, 41). Novartis' MF59 and GlaxoSmithKline's AS03 are both vaccine adjuvants that consist of oil-in-water emulsions,

comprising of 4.3% of squalene oil as the dispersed oil phase, which is stabilized by two non-ionic surfactants (Tween 80 and Span 85), and a low ionic strength citrate buffers as continuous phase (51, 52). AS04 contains 3-O-desacyl-40-monophosphoryl lipid A (MPL) derived from the lipopolysaccharide (LPS) of *Salmonella minnesota*, R595 (53) and aluminum hydroxide or aluminum phosphate in a 1 to 10 ratio (w/w) (52). AF03 is a novel squalene-in-water emulsion manufactured by a phase inversion temperature emulsification process and is recently approved in human influenza vaccine Humenza™ (54, 55). Virosomes are a biodegradable and non-toxic adjuvant system that contains virus envelopes devoid of inner core and genetic materials (56). Virosomal adjuvant system induces both B- and T-cell responses and is approved in variety of vaccines (57). Montanide™ ISA51 is composed of a light mineral oil and a surfactant designed to make a water-in-oil emulsion (58).

1.5 APPROACHES TO OVERCOME THE LIMITATIONS

There is great interest in addressing this problem, and the strategies to solve it are generally two-fold. The first is to add stabilizing reagents in the vaccines to prevent aggregation during freezing. For example, the Program for Appropriate Technology (PATH) and its research collaborators have shown that adding glycerin, polyethylene glycol 300, or propylene glycol into vaccines adjuvanted with aluminum salts prevents vaccine aggregation and preserves vaccine efficacy, even after the vaccine is subjected to multiple exposures to -20° C (59). Zapata *et al*. also reported that the adsorption of polymers or surface-active agents, such as hydroxypropyl methylcellulose, sodium lauryl

sulfate, and polysorbate 80, on aluminum hydroxide prevents aggregation after a freeze-thaw cycle (60). It is thought that the stabilizing agents produce a large steric repulsive region between particles and hinder particle-particle interaction (60). The strength of the repulsive region formed by stabilizing agents is proportional to their molecular length (60). However, the addition of the aforementioned excipients into vaccine may result in a more complex formulation and increase the cost per dose of the vaccine.

Another strategy is to convert aluminum salt-adjuvanted vaccines into a solid form using novel freezing and/or drying techniques. Various methods, such as vacuum-foam drying (61), spray drying (62), spray freeze-drying (63), and spray freezing into liquid (64), have been previously explored to convert protein products into dry powders. Spray freeze-drying has been extensively studied for freeze-drying vaccines that contain aluminum salts (65, 66). Maa *et al*. suspended an aluminum hydroxide-adjuvanted hepatitis-B surface antigen (Alum-HBsAg) vaccine into mannitol, glycine, and dextran, with final aluminum hydroxide concentration adjusted to 3% or 0.6% (w/v), sprayed atomized liquid droplets (within the range of 20–80 μm) of the Alum-HBsAg vaccine into a liquid nitrogen-containing pan, and then placed the pan containing frozen particles in liquid nitrogen to a precooled (−55° C) shelf freeze dryer to lyophilize the particles into a dry powder (63). After reconstitution, the size of the particles in the Alum-HBsAg vaccine did not increase (63). The authors also evaluated the anti-HBsAg IgG titers induced by the reconstituted Alum-HBsAg vaccines in mice and compared them with the original untreated Alum-HBsAg vaccine. Unfortunately, the reconstituted vaccines were administered by intraperitoneal injection and the untreated Alum-HBsAg vaccine was

injected intramuscularly (63), making it impossible to conclude whether the spray freeze-dried vaccines were as immunogenic as the untreated vaccine. Nonetheless, the formulations that contained 3% (w/v) of aluminum hydroxide induced an anti-HBsAg IgG titer that was close to 10-fold less than that induced by the untreated Alum-HBsAg vaccine, and the anti-HBsAg IgG titer induced by the formulation with 0.6% of aluminum hydroxide was about 2-fold less than that induced by the untreated Alum-HBsAg vaccine (63). It was concluded that lowering alum concentration and fast freezing are the most effective formulation parameters in minimizing particle coagulation and thus maximizing the immunogenicity of the vaccine (63).

Clausi *et al*. used a similar spray freeze-drying method to dry lysozyme or alkaline phosphatase adjuvanted with aluminum hydroxide (Alhydrogel) or aluminum phosphate (67, 68). The lysozyme vaccine formulations were prepared consisting of 25 mM sodium succinate (pH 4.0), 10 μg/ml lysozyme, 0.2 w/v% of either aluminum hydroxide or aluminum phosphate, and 0, 2, or 7.5 w/v% trehalose (68). The vaccines were spray-frozen by dropping ~20 μl droplets into liquid nitrogen and then lyophilized. As a control, the vaccines were also slowly tray-frozen by placing the samples in lyophilizer, equilibrating 1 h at a shelf temperature of 0°C, then cooling the shelves at 0.5° C/min to −40° C ("tray-freezing") (68). The size of the particles in the reconstituted vaccines after tray freeze-drying or spray freeze-drying was generally larger than the size of the particles in the originally prepared formulation (68). All of the lyophilized formulations were easily reconstituted with water, and the adjuvant particles were well dispersed upon gentle shaking. However, noticeable settling of the lyophilized vaccines was observed within a

few minutes of sitting, especially in formulations without trehalose (vs. about 30 min for all untreated liquid vaccines) (68). Interestingly, when subcutaneously administered into mice, the vaccines reconstituted from the freeze-dried vaccines all induced a stronger anti-lysozyme IgG1 response than the untreated liquid vaccine (68). It was concluded that higher cooling rates and higher excipient levels minimize adjuvant agglomeration (68).

The alkaline phosphatase vaccine formulation used in Clausi and coworkers' study was prepared containing 0.081 mg/mL alkaline phosphatase, 0.2% (w/v) of aluminum hydroxide, 7.5% (w/v) trehalose, and 25 mM Tris (pH 7.5) (67). The vaccine was then spray freeze-dried or tray freeze-dried as mentioned above. The size of the particles in the originally prepared liquid vaccine peaked around 1 μm, but the sizes of the particles in the vaccine reconstituted from the spray freeze-dried and tray freeze-dried vaccines peaked around 4-5 μm and 10-20 μm, respectively (67). All alkaline phosphatase vaccines, liquid or reconstituted from freeze-dried powders, induced similar levels of alkaline phosphatase specific IgG1 response in mice (67).

Spray freeze-drying and tray freeze-drying were also applied to a botulism vaccine candidate containing botulinum neurotoxin subtype E (BoNT/E), aluminum hydroxide (0.2%, w/v), and trehalose (10%, w/v) (69). When tested in mice, the anti-BoNT/E IgG1 titers in mice injected with reconstituted spray freeze-dried or tray freeze-dried vaccines appeared to be lower, and more variable, than that induced by the untreated liquid vaccine, especially when measured after a booster immunization, although it was stated that the differences were not significant (69).

Thin-film freezing (TFF) has been recently studied in the biopharmaceutical field for preparing stable submicron protein particles (70). In the TFF process, a liquid (e.g., solution) is spread out on a cryogenic substrate to form a thin film in less than one second. The resultant frozen film is then dried by lyophilization. For example, Engstrom *et al*. produced dried protein powders with a diameter of 300 nm using TFF, and the enzyme activity of the proteins was fully preserved (70). In the present study, the feasibility of freeze-drying vaccines that are adjuvanted with aluminum salts using TFF was tested. Ovalbumin (OVA) was initially used as a model protein antigen, and it was adjuvanted with aluminum hydroxide or aluminum phosphate and lyophilized after thin-film freezing. In addition, a commecially availble veterinary tetanus toxoid vaccine (tetanus antitoxin concentrated/purified, Colorado Serum Company, Denver, CO), a human hepatitis B vaccine (Engerix-B, GlaxoSmithKline Biologics), and a human papillomavirus quadrivalent vaccine (Gardasil, Merck & Co.) were also successfully converted into dry powders using the thin-film freeze-drying (TFFD) method. It was concluded that TFFD can be used to convert vaccines that are adjuvanted with aluminum salts into dry powder, without causing particle aggregation or decreasing the immunogenicity of the vaccines.

1.6 NEEDLE-FREE IMMUNIZATION

Immunization is one of the most powerful and cost-effective of all health interventions (71). It averts an estimated 2.5 million deaths a year. Over 100 million children are immunized every year but despite the success, millions of children in developing countries- almost 20% of all the children born every year (around 24 million)

- do not get the complete immunizations scheduled for their first year of life (71).Vaccines are given mostly by injections. WHO estimates that around 12 billion injections are given every day out of which 5% are used for immunizations (72). Fear of needles is critical issue for both adults and children (73). Besides that, accidental needle sticks are a serious problem in both developed and developing countries. Even greater shortcoming is improper and unsafe use of needles (72). According to WHO, one-third of immunization injections are unsafe in four of the six geographical regions (71). Another risk of needle-stick injury is transmission of the blood-borne pathogens (74). Development of needle-free immunization has been identified as an important goal in global health care (75).

The problems associated with vaccine stability urges new vaccine formulations with better stability. Approaches for needle-free immunization include cutaneous and mucosal immunization. Cutaneous methods of immunization include following: liquid-jet injection, which uses high pressure narrow jet of the liquid vaccine stream into intradermal, subcutaneous or intramuscular regions (76); epidermal powder immunization which is a ballistic method that can efficiently deliver powdered vaccines to the epidermis (77). Mucosal immunization methods involve delivery of vaccines to mucosal membranes such as ocular, nasal, pulmonary or rectal membrane. Oral delivery of non-living vaccines has proved to be extremely challenging due to deactivation of the vaccine in the acidic environment of the gastrointestinal tract (78). Vaginal or rectal immunizations suffers disadvantages of poor patient compliance. Given that many pathogens invade the host via mucosal membrane, nasal and pulmonary routes are attractive site for immunization (79).

1.7 POWDER VACCINES FOR INTRANASAL DELIVERY

Aluminum salt-adjuvanted vaccines are generally administered by subcutaneous, intradermal, or intramuscular injection. However, reconstituting the dry powder vaccine before injection has various limitations such as the need for sterile water for injection, the need for trained medical personnel, and the increased chance of error made when reconstituting the powder and filling syringes. The nasal route for immunization offers some interesting opportunities. Almost all infectious agents enter the body through the mucosal surfaces (80), and the nasal mucosa is often the first point of contact for inhaled pathogens. Therefore, ideally, to more effectively protect against inhaled pathogens, vaccines should be administered via the nasal mucosal surface to induce mucosal immunity to prevent infectious agents from entering the host (81). Besides enabling vaccines to induce both mucosal and systemic immune responses (82, 83), intranasal immunization has several other advantages as well. For example, the nose tissue is easily accessible and highly vascularized, and can be used in the case of epidemics for mass vaccination. In addition, nasal immunization enables needle-free, non-invasive delivery of vaccines with the possibility of self-immunization.

Despite the demonstrated efficacy and advancement in the science of nasal delivery technology, there are no commercially available nasal dry powder vaccines in the market. The major barrier lies in the development of appropriate formulations for these antigen candidates. First of all, we need a stable formulation of antigen with adjuvant. Antigens are attached with adjuvants with either physical adsorption, or covalently attached. Some adjuvants are added with antigen without any attachment. Aluminum salts are attached

with antigens using physical adsorption. So, any type of physical strain makes the weak bonds susceptible to break. The challenge lies in making a stable dry vaccine powder formulation without affecting stability of the antigens or without affecting the tertiary and quaternary structure of the antigen.

For example, during SD and SFD, the material is subjected to stresses that may be harmful to the antigenicity of the vaccine (84). In SD, it is atomization stress, drying stress and dehydration stress, where as in SFD, it is atomization stress, freezing stress and dehydration stress (85-87). Also, size reduction technology, such as jet mill or pearl mill, can generate heat and chances are there that the antigens is detached from the adjuvant. Several studies have shown that these stresses can be prevented by adding sugars to the liquid feed (88-90). Sugars can form glassy matrix, if properly dried (91). Incorporation of proteins in sugar glass can also increase the shelf life. Mechanism of stabilization by sugar glasses: (1) the glassy sugar matrix can reduce the mobility of entrapped biopharmaceuticals (92). (2) Sugars act as a physical barrier to prevent aggregation between two biopharmaceutical molecule (93). (3) Sugar molecules replace water molecules that form hydrogen bonds with the biopharmaceuticals (90). A good stabilizer should have low number of reducing groups and high glass transition temperature.

Previously, it was thought that aluminum salt-based adjuvants are not capable of potentiating mucosal immune responses when given intranasally, and results from prior studies were ambiguous (94-97). However, data from our recent studies clearly showed that intranasal immunization with antigens adsorbed on Alhydrogel® induces significantly

stronger antigen-specific immune responses, both systemically and in nasal and lung mucosa, as compared to intranasal immunization with the antigens alone (98).

1.8 OBJECTIVES

As mentioned earlier in section 1.4, unintentional freezing during transport and storage is a common place and not resource limited. According to WHO, the vaccines that have been exposed to freezing temperatures accidentally, have to be discarded. This in effect causes costly waste of global vaccine supplies and/or the widespread delivery of vaccines with compromised potency. The motivation of this dissertation is to overcome this significant unmet need of the insoluble aluminum salt-adjuvanted vaccines in the vaccine industry. For the first part, we present here a novel freeze-drying method to prepare stable formulations of the aluminum salt-adjuvanted vaccines. Furthermore, there is an increasing interest in novel approaches to vaccine stability management such as the controlled temperature chain (CTC) storage, which allows vaccines to be managed in temperatures outside of the traditional cold-chain for a limited period. To test that, long term thermal stability and the freeze stability of the dry powder formulation was evaluated. At last, the nasal delivery of these stable dry powder formulations which enables needle-free, noninvasive delivery of vaccines with a possibility of self-immunization was tested. The specific hypothesis of each studies are as follows:

- **To test the feasibility of freeze-drying vaccines that are adjuvanted with insoluble aluminum salts using a novel ultra-rapid freezing technique called thin-film freeze-drying (TFFD).** In this study, six different aluminum salt-

adjuvanted vaccines and five different aluminum-containing adjuvants are tested to evaluate the aggregation of the aluminum salt after converting to dry powder vaccine. Out of those, two dry powder vaccine formulations are compared to evaluate the antibody responses in a mouse model (Chapter two).

- **To evaluate the thermal and freeze stability of a thin-film freeze-dried (TFFD) aluminum salt adjuvanted dry powder vaccine formulations.** In this study, the immunogenicity of the dry vaccine powder formulation was evaluated in temperatures as high as 40 ºC for up to 6 months, as well as after the dry powder vaccine formulation was subjected to repeated slow freezing-and-thawing cycles (Chapter three).
- **To evaluate the systemic and mucosal antibody responses induced by dry powder vaccine of aluminum salt adjuvanted vaccines when administered intranasally.** In this study, a novel dry powder delivery device was invented. Also, the dry powder vaccine formulation of aluminum salt adjuvanted vaccine was further characterized to determine its feasibility towards intranasal immunization (Chapter four).

Chapter Two

A method of lyophilizing vaccines containing aluminum salts into a dry powder without causing particle aggregation or decreasing the immunogenicity following reconstitution[1]

2.1 INTRODUCTION

Some aluminum salts, including aluminum hydroxide and aluminum phosphate, have been widely used as human vaccine adjuvants for decades. The primary particles of aluminum hydroxide and aluminum phosphate are in the nanometer-scale. However, when dispersed in an aqueous solution, the primary particles aggregate to form larger microparticles of 1-20 μm (99, 100). Thus, a vaccine that is prepared by binding an antigen with an aluminum salt is physically a suspension of aluminum salt particles with antigens adsorbed on them. Three mechanisms are frequently cited to explain the mechanisms underlying the adjuvant activity of aluminum salts (35, 36, 100-102): i) for decades, it was thought to be the depot effect. Aluminum salts form an antigen depot at the injection site, from where the antigens are slowly released (103); ii) Aluminum salts induce inflammation, thus recruiting and activating antigen-presenting cells that capture antigens (104); iii) The adsorption of soluble antigens on aluminum salt particles makes them

[1]This Chapter is based on "**Sachin G. Thakkar**[*], Xinran Li[*], Tinashe B Ruwona, Robert Williams III, Zhengrong Cui, ***Journal of Controlled Release***, *April 2015, 204: 38–50.* [*]authors contributed equally.

readily taken up by antigen-presenting cells (104). Finally, recent data showed that the molecular target for the pro-inflammatory activity of aluminum salts is the NOD-like receptor protein 3 (NLRP3) (104-108).

Many currently licensed and commercially available vaccines, including diphtheria-tetanus-pertussis vaccines, Hepatitis A vaccines, Hepatitis B vaccines, Pneumococcal conjugate vaccines, anthrax vaccine, human papillomavirus vaccine, and Rabies vaccines, contain aluminum salts as adjuvants (109). However, a major limiting factor with these vaccines is that they must not be exposed to freezing conditions during transport and storage, and are too fragile to be stable at ambient temperatures. In other words, vaccines that are adjuvanted with aluminum salts must remain stored as a liquid suspension at 2-8^{o} C from manufacturing to being administered to patients, because inadvertently exposing the suspension to freezing temperatures causes irreversible coagulation that damages the vaccines (e.g., loss in activity and stability) (59). Vaccines that have been incidentally exposed to freezing conditions before administration to patients must be discarded, causing significant product waste and limited utility. This is significant considering that an estimated 75-100% of the vaccine shipments are actually exposed to freezing temperatures at some points during shipment (110), resulting in costly waste and the loss of nearly half of all global vaccine supplies (46).

There is great interest in addressing this problem, and the strategies to solve it are generally two-fold. The first is to add stabilizing reagents in the vaccines to prevent aggregation during freezing. For example, the Program for Appropriate Technology (PATH) and its research collaborators have shown that adding glycerin, polyethylene

glycol 300, or propylene glycol into vaccines adjuvanted with aluminum salts prevents vaccine aggregation and preserves vaccine efficacy, even after the vaccine is subjected to multiple exposures to -20° C (59). Zapata *et al*. also reported that the adsorption of polymers or surface-active agents, such as hydroxypropyl methylcellulose, sodium lauryl sulfate, and polysorbate 80, on aluminum hydroxide prevents aggregation after a freeze-thaw cycle (60). It is thought that the stabilizing agents produce a large steric repulsive region between particles and hinder particle-particle interaction (60). The strength of the repulsive region formed by stabilizing agents is proportional to their molecular length (60). However, the addition of the aforementioned excipients into vaccine may result in a more complex formulation and increase the cost per dose of the vaccine.

Another strategy is to convert aluminum salt-adjuvanted vaccines into a solid form using novel freezing and/or drying techniques. Various methods, such as vacuum-foam drying (61), spray drying (62), spray freeze-drying (63), and spray freezing into liquid (64), have been previously explored to convert protein products into dry powders. Spray freeze-drying has been extensively studied for freeze-drying vaccines that contain aluminum salts (65, 66). Maa *et al*. suspended an aluminum hydroxide-adjuvanted hepatitis-B surface antigen (Alum-HBsAg) vaccine into mannitol, glycine, and dextran, with final aluminum hydroxide concentration adjusted to 3% or 0.6% (w/v), sprayed atomized liquid droplets (within the range of 20–80 μm) of the Alum-HBsAg vaccine into a liquid nitrogen-containing pan, and then placed the pan containing frozen particles in liquid nitrogen to a precooled (−55° C) shelf freeze dryer to lyophilize the particles into a dry powder (63). After reconstitution, the size of the particles in the Alum-HBsAg vaccine

did not increase (63). The authors also evaluated the anti-HBsAg IgG titers induced by the reconstituted Alum-HBsAg vaccines in mice and compared them with the original untreated Alum-HBsAg vaccine. Unfortunately, the reconstituted vaccines were administered by intraperitoneal injection and the untreated Alum-HBsAg vaccine was injected intramuscularly (63), making it impossible to conclude whether the spray freeze-dried vaccines were as immunogenic as the untreated vaccine. Nonetheless, the formulations that contained 3% (w/v) of aluminum hydroxide induced an anti-HBsAg IgG titer that was close to 10-fold less than that induced by the untreated Alum-HBsAg vaccine, and the anti-HBsAg IgG titer induced by the formulation with 0.6% of aluminum hydroxide was about 2-fold less than that induced by the untreated Alum-HBsAg vaccine (63). It was concluded that lowering alum concentration and fast freezing are the most effective formulation parameters in minimizing particle coagulation and thus maximizing the immunogenicity of the vaccine (63).

Clausi *et al*. used a similar spray freeze-drying method to dry lysozyme or alkaline phosphatase adjuvanted with aluminum hydroxide (Alhydrogel) or aluminum phosphate (67, 68). The lysozyme vaccine formulations were prepared consisting of 25 mM sodium succinate (pH 4.0), 10 µg/ml lysozyme, 0.2 w/v% of either aluminum hydroxide or aluminum phosphate, and 0, 2, or 7.5 w/v% trehalose (68). The vaccines were spray-frozen by dropping ~20 µl droplets into liquid nitrogen and then lyophilized. As a control, the vaccines were also slowly tray-frozen by placing the samples in lyophilizer, equilibrating 1 h at a shelf temperature of 0°C, then cooling the shelves at 0.5° C/min to −40° C ("tray-freezing") (68). The size of the particles in the reconstituted vaccines after tray freeze-

drying or spray freeze-drying was generally larger than the size of the particles in the originally prepared formulation (68). All of the lyophilized formulations were easily reconstituted with water, and the adjuvant particles were well dispersed upon gentle shaking. However, noticeable settling of the lyophilized vaccines was observed within a few minutes of sitting, especially in formulations without trehalose (vs. about 30 min for all untreated liquid vaccines) (68). Interestingly, when subcutaneously administered into mice, the vaccines reconstituted from the freeze-dried vaccines all induced a stronger anti-lysozyme IgG1 response than the untreated liquid vaccine (68). It was concluded that higher cooling rates and higher excipient levels minimize adjuvant agglomeration (68).

The alkaline phosphatase vaccine formulation used in Clausi and coworkers' study was prepared containing 0.081 mg/mL alkaline phosphatase, 0.2% (w/v) of aluminum hydroxide, 7.5% (w/v) trehalose, and 25 mM Tris (pH 7.5) (67). The vaccine was then spray freeze-dried or tray freeze-dried as mentioned above. The size of the particles in the originally prepared liquid vaccine peaked around 1 μm, but the sizes of the particles in the vaccine reconstituted from the spray freeze-dried and tray freeze-dried vaccines peaked around 4-5 μm and 10-20 μm, respectively (67). All alkaline phosphatase vaccines, liquid or reconstituted from freeze-dried powders, induced similar levels of alkaline phosphatase specific IgG1 response in mice (67).

Spray freeze-drying and tray freeze-drying were also applied to a botulism vaccine candidate containing botulinum neurotoxin subtype E (BoNT/E), aluminum hydroxide (0.2%, w/v), and trehalose (10%, w/v) (69). When tested in mice, the anti-BoNT/E IgG1 titers in mice injected with reconstituted spray freeze-dried or tray freeze-dried vaccines

appeared to be lower, and more variable, than that induced by the untreated liquid vaccine, especially when measured after a booster immunization, although it was stated that the differences were not significant (69).

Thin-film freezing (TFF) has been recently studied in the biopharmaceutical field for preparing stable submicron protein particles (70). In the TFF process, a liquid (e.g., solution) is spread out on a cryogenic substrate to form a thin film in less than one second. The resultant frozen film is then dried by lyophilization. For example, Engstrom *et al*. produced dried protein powders with a diameter of 300 nm using TFF, and the enzyme activity of the proteins was fully preserved (70). In the present study, the feasibility of freeze-drying vaccines that are adjuvanted with aluminum salts using TFF was tested. Ovalbumin (OVA) was initially used as a model protein antigen, and it was adjuvanted with aluminum hydroxide or aluminum phosphate and lyophilized after thin-film freezing. In addition, a commecially availble veterinary tetanus toxoid vaccine (tetanus antitoxin concentrated/purified, Colorado Serum Company, Denver, CO), a human hepatitis B vaccine (Engerix-B, GlaxoSmithKline Biologics), and a human papillomavirus quadrivalent vaccine (Gardasil, Merck & Co.) were also successfully converted into dry powders using the thin-film freeze-drying (TFFD) method. It was concluded that TFFD can be used to convert vaccines that are adjuvanted with aluminum salts into dry powder, without causing particle aggregation or decreasing the immunogenicity of the vaccines.

2.2 MATERIALS AND METHODS

2.2.1 Materials

Dried aluminum hydroxide gel (AH gel) and aluminum phosphate were from Spectrum Chemical and Laboratory Products (Gardena, CA). OVA, trehalose, and Laemmli sample buffer were from Sigma-Aldrich (St. Louis, MO). Bio-safe™ Coomassie blue staining solution and Bio-Rad DC™ protein assay reagents were from Bio-Rad Laboratories (Hercules, CA). Alhydrogel® (2%, w/v) was from InvivoGen (San Diego, CA). Tetanus antitoxin concentrated/purified (TT vaccine) was from Colorado Serum Company (Denver, CO). The TT vaccine contains potassium alum (personal communication with Dr. Randall Berrier at Colorado Serum). Potassium alum is also known as potassium aluminum sulfate. Engerix-B, a human hepatitis B vaccine from GlaxoSmithKline, and Gardasil, a human papillomavirus quadrivalent vaccine from Merck & Co., Inc., were purchased through the University of Texas at Austin University Health Services. Engerix-B contains aluminum hydroxide (0.5 mg of aluminum per ml). Gardasil contains amorphous aluminum hydroxyphosphate sulfate (0.45 mg/ml) as an adjuvant. Mouse Anti-Tetanus Toxoid Ig's ELISA kit was from Alpha Diagnostic International (San Antonio, TX). Tetanus toxoid was from List Biologics Laboratory (Campbell, CA). Purified polyclonal horse anti-tetanus serum and guinea pig anti-tetanus IgG were from the National Institute for Biological Standards and Control (Hertfordshire, England).

2.2.2 Thin-film freeze-drying (TFFD)

Three types of aluminum-containing compounds, dried aluminum hydroxide gel (USP grade) (AH gel), 2% Alhydrogel®, and aluminum phosphate, were used to adsorb OVA as a model antigen. The OVA-adsorbed aluminum hydroxide vaccine was prepared by mixing an OVA solution with an aluminum hydroxide suspension in phosphate buffered saline (PBS, pH 7.4, 10 mM) to reach an OVA to Al^{3+} weight ratio of 1:10. The vaccine contained 31.4 μg/ml of OVA, 0.09% of aluminum hydroxide, and 0-5% (w/v) of trehalose. The OVA-aluminum phosphate vaccine (31.4 μg/ml of OVA, 0.142% (w/v) of aluminum phosphate, and 2% (w/v) of trehalose) was prepared similarly. When the 2% Alhydrogel® was used, Alhydrogel® (25 ml) was added into a 50 mL tube, followed by the addition of 25 ml of an OVA solution (1 mg/ml) at an OVA to Al^{3+} weight ratio of 1:10, and 1 g of trehalose to obtain a final formulation with 2% (w/v) of trehalose, ~1% (w/v) of Alhydrogel®, and 0.5 mg/mL of OVA. The samples were subjected to TFF and lyophilized as described previously (70, 111). Briefly, the aluminum-containing vaccine suspensions were dropped onto a pre-cooled rotating cryogenic steel surface to form thin films. The thin films were removed by a steel blade. In order to avoid the overlap of two droplets, the speed at which the vaccine suspension was dropped on the cryogenic substance was controlled at 5-7 rpm. The frozen film-like solids were collected in liquid nitrogen and dried using a VirTis Advantage bench top tray lyophilizer (The VirTis Company, Inc. Gardiner, NY). Lyophilization was performed over 72 h at pressures less than 200 mTorr, while the shelf temperature was gradually ramped from −40°C to 26° C. After

lyophilization, the solid vaccine powder was quickly transferred to a sealed container and stored in a desiccator at room temperature before further use (112).

To dry TT vaccine, trehalose was added into the TT vaccine that was diluted 50-fold in PBS (pH 6.3, 10 mM) to adjust the final concentration of trehalose to 2% (w/v). The vaccine was then subjected to TFFD as mentioned above. To dry Engerix-B, trehalose was added directly into the commercial vaccine (without pre-dilution) to obtain a formulation with 2% (w/v) of trehalose, ~20 μg/mL of HBsAg, and ~500 μg/mL of aluminum, and the vaccine was then subjected to TFFD. In Engerix-B vaccine, each 1-mL adult dose contains 20 μg of HBsAg adsorbed on 0.5 mg of aluminum as aluminum hydroxide. To dry the Gardasil vaccine, 100 μL of the vaccine was diluted to 1 mL of 0.9% (w/v) sodium chloride, and trehalose was added to reach a final concentration of 2% (w/v). The vaccine was then subjected to TFFD. Each 0.5-mL dose of the original Gardasil contains approximately 20 μg of HPV 6 L1 protein, 40 μg of HPV 11 L1 protein, 40 μg of HPV 16 L1 protein, and 20 μg of HPV 18 L1 protein. Each 0.5-ml dose of Gardasil also contains approximately 225 μg of aluminum (as amorphous aluminum hydroxyphosphate sulfate).

The morphology of the vaccines in suspension was examined under an Olympus BX60 microscope (Olympus America, Inc., Center Valley, PA). The size of particles and particle size distribution in all samples were determined using a Sympatec Helos laser diffraction instrument (Sympatec GmbH, Germany) equipped with a R3 lens. The moisture in the dried powder was measured using a Karl Fisher Titrator Aquapal III from CSC Scientific Company (Fairfax, VA).

2.2.3 Shelf freeze-drying

An OVA-adsorbed aluminum hydroxide (AH gel) vaccine that contained 2% of trehalose (w/v), 0.09% of aluminum hydroxide, and 31.4 μg/mL of OVA in PBS (pH 7.4, 10 mM) was frozen on the shelf of a -20ºC or -80ºC freezer overnight and then lyophilized using a VirTis Advantage bench top tray lyophilizer as mentioned above. The dry powder was stored in a desiccator at room temperature before use.

2.2.4 The effect of the concentration of trehalose in vaccine on thin-film freeze-drying

To evaluate the effect of the concentration of trehalose on TFFD of vaccines, various amounts of trehalose were added into OVA-adsorbed aluminum hydroxide (AH gel) in suspension (1:10, OVA vs. Al^{3+}, w/w) to prepare formulations with 0%, 1%, 2%, 3%, 4%, and 5% of trehalose (w/v). The suspensions were then subjected to TFFD as mentioned above.

2.2.5 The binding efficiency of OVA to the aluminum hydroxide before and after TFFD

SDS-PAGE was used to determine the binding efficiency of OVA to aluminum hydroxide before and after TFFD (41). The OVA-adsorbed aluminum hydroxide (AH gel) dry powder (OVA to Al^{3+} ratio, 1 to 10, w/w) was reconstituted and applied on SDS-PAGE gel. As a control, OVA alone and freshly prepared OVA-adsorbed aluminum hydroxide suspension (with 2% trehalose, w/v) were also included. Samples were mixed with a Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, and 0.01%

Bromophenol Blue) before applied to 7.5% Mini-PROTEAN® TGX™ precast polyacrylamide gels (Bio-Rad). Precision plus protein standards were also run along with the samples at 130 V for 1 h. The gel was then stained in a Bio-Safe™ Coomassie blue staining solution and scanned using a Kodak Image Station 440CF (Rochester, NY). The intensity of the protein bands in the gel was quantified using the NIH ImageJ software, and the binding efficiency was calculated by subtracting the percentage of unbound protein (i.e., band intensity from vaccine dry powder or freshly prepared vaccine suspension) from the total protein (i.e., band intensity of OVA alone).

The binding efficiency of the OVA to aluminum hydroxide (AH gel) was also determined by centrifuging the OVA-adsorbed aluminum hydroxide, before and after TFFD and reconstitution, at about 4500 x rcf for 5 min, and measuring the concentration of the OVA in the supernatant using the BCA Protein Assay Reagent from Thermo Scientific (Pittsburgh, PA) (113). The amount of OVA bound on the aluminum hydroxide was calculated by subtracting the amount of OVA in the supernatant from the total amount of OVA added into the vaccine.

2.2.6 Differential scanning calorimetry (DSC)

Thermal analyses of the OVA-adsorbed aluminum hydroxide (AH gel) dry powder and its individual components, OVA, aluminum hydroxide (AH gel), and trehalose, were conducted using a modulated temperature DSC (Model 2920, TA Instruments, New Castle, DE) (111). Four to seven milligrams of each sample was weighed into the aluminum pans (PerkinElmer Instruments, Norwalk, CT), which were crimped subsequently. An empty

aluminum pan was used as a reference. Samples were then heated at a ramp rate of 3°C/min from -30°C to 300°C. Data were analyzed using the TA Universal Analysis 2000 software (TA Instruments).

2.2.7 Scanning electron microscopy (SEM)

The morphology of the OVA-adsorbed aluminum hydroxide (AH gel) dry powder and freshly prepared OVA-adsorbed aluminum hydroxide (AH gel) suspension was examined using a Zeiss Supra 40 VP scanning electron microscope in the Institute for Cell and Molecular Biology Microscopy and Imaging Facility at The University of Texas at Austin (114). When preparing the TFFD samples for SEM, one thin layer of the dried powder was deposited on the specimen stub using a double stick carbon tape. For the freshly prepared OVA-adsorbed aluminum hydroxide suspension, the suspension was placed on the specimen stub and allowed to dry overnight. The specimen stubs with samples were then placed in the sputter coater chamber and coated with a very thin film of lead (Pb) before examination.

2.2.8 Intrinsic tryptophan fluorescence spectrometry

The TT vaccine was dried using TFFD and reconstituted before examination. Freshly diluted TT vaccine (in a phosphate buffer) was used as a negative control. The final trehalose concentration in both samples was 2% (w/v). Fluorescence emission spectrum was recorded using a PTI Quanmaster spectrofluorimeter (Photon Technology International, Santa Clara, CA). An excitation wavelength of 290 nm was employed, and the emission spectrum was collected from 280 nm to 530 nm (115).

2.2.9 Repeated freeze-thawing of thin-film freeze-dried vaccine powder

The dried powders of TT vaccine, Engerix B, and OVA-adsorbed Alhydrogel® were subjected to three cycles of freezing (-20ºC for 8 h) and thawing (4ºC for 16 h), reconstituted, and analyzed for particle size distribution. As control, fresh vaccines were also subjected to the same three cycles of freezing-and-thawing and examined similarly.

2.2.10 Animal studies

All animal studies were carried out following the National Research Council guide for the care and use of laboratory animals. The animal protocol was approved by the Institutional Animal Care and Use Committee at The University of Texas at Austin. Female BALB/c mice, 6-8 weeks of age, were from Charles River Laboratories, Inc. (Wilmington, MA). Mice (n = 5) were subcutaneously (s.c.) injected with OVA-adsorbed aluminum hydroxide (AH gel) or the TT vaccine, freshly prepared or reconstituted from TFFD powder. For the OVA-adsorbed aluminum hydroxide (AH gel), mice were immunized on days 0, 14 and 28 with 5 μg, 10 μg, or 20 μg of OVA per mouse. As controls, mice were injected with sterile PBS or OVA alone (10 μg) dissolved in PBS. For the TT vaccine, mice were immunized on days 0, 14, and 28, and the dose of TT was 3.75 Lf (flocculation units) of tetanus toxoid per mouse per injection. Sterile PBS and TT vaccine freshly diluted with 2% trehalose were used as controls. Sixteen days after the third dose, mice were bled for antibody assay. Total anti-OVA IgG levels in serum samples were measured using enzyme-linked immunosorbent assay (ELISA) as described previously (116). Anti-TT IgG levels

were determined using a mouse Anti-Tetanus Toxoid Ig's ELISA Kit following the manufacturer's instructions.

2.2.11 Tetanus toxin binding inhibition test (ToBI-test)

The ToBI-test is an *in vitro* assay validated to determine the activity of anti-tetanus toxoid antiserum in vaccination studies and was adapted (117-119). Briefly, flat bottom microplates (Corning Costar, NY, NY) (PI) were blocked for 90 min at 37°C in a humidified chamber with 250 µl/well PBS containing 3% bovine serum albumin (BSA, Sigma-Aldrich) and 0.05% Tween 20 (PBS-BSA). After washing with PBS containing 0.05% Tween 20 (PBS-T), mouse serum samples diluted 2-fold serially in Peptone diluent (i.e., 1 % peptone, 0.5% NaCl, 0.5% BSA and 0.05% Tween 20) were added into the wells (duplicate in 100 µl, starting at 1:200 dilution). Wells, in which serum samples were not added, were used as the 100% binding signal in calculating the percent binding. Subsequently, 100 µl tetanus toxoid (0.65 µg/ml diluted in 1% peptone and 0.5% Tween 20) was added into all wells. Wells without tetanus toxoid were also included as a control. These microplates were then incubated overnight. A parallel series of plates (Maxisorp® Nunc,Thermo Scientific) (P2) were coated with 2 IU/ml of purified polyclonal horse anti-tetanus serum (NIBSC 60/013, diluted in PBS, 10 mM, pH 7.4) and incubated overnight. The next day, these plates were blocked for 90 min with 200 µl per well of PBS-BSA. After washing with PBS-T, 100 µl of serum-toxoid mixtures in the P1 microplates were transferred to the wells in the P2 microplates and incubated for 90 min. After a third washing step, 100 µl guinea pig anti-tetanus IgG (NIBSC 10/132) was added to the wells

(1:200 dilution in PBS-BSA) and incubated for 90 min. After washing with PBS-T, 100 µl of peroxidase-conjugated goat anti-guinea pig IgG (Sigma-Aldrich) at 1:2000 dilution was added and incubated for 60 min. One hundred microliters of 3,3′,5,5′-Tetramethylbenzidine (TMB) Liquid Substrate System for ELISA (Sigma-Aldrich) was added and incubated in the dark for 15 min. The reaction was stopped by the addition of 100 µl of 2N H_2SO_4, and the absorbance was measured at 450 nm. The percentage of binding (of tetanus toxoid to the polyclonal horse anti-tetanus antiserum coated in P2 microplates) was reported as the OD450 values of the samples as a percentage of the OD450 values in wells with zero percent of inhibition (i.e., tetanus toxoid that was not mixed with any mouse antiserum samples).

2.2.12 Statistics

Statistical analyses were conducted using analysis of variance followed by Fischer's protected least significant difference procedure. A p-value of ≤ 0.05 (two-tail) was considered statistically significant.

2.3 RESULT AND DISCUSSION

2.3.1 Thin-film freeze-drying of OVA-adsorbed aluminum hydroxide

In order to test whether the TFFD can be used to lyophilize an aluminum hydroxide-adjuvanted, protein-based vaccine, OVA-adsorbed aluminum hydroxide was suspended in 2% (w/v) of trehalose and subjected to TFFD. A white powder was formed, which can be readily reconstituted with water, PBS, or normal saline with no or only minimal agitation. The moisture content in the powder was 1-3%. The size of the particles in the reconstituted OVA-adsorbed aluminum hydroxide was 9.7 ± 2.5 μm, which is not different from the size of the particles in freshly prepared OVA-adsorbed aluminum hydroxide suspension (9.4 ± 1.7 μm) (Fig. 1A), demonstrating that the OVA-adsorbed aluminum hydroxide suspension can be successfully lyophilized into a dry powder form using TFFD without significantly affecting on the size of the particles in the vaccine. The microscopic images in Figs. 1B-C also show that subjecting the OVA-adsorbed aluminum hydroxide to TFFD and reconstitution did not cause significant aggregation. In contrast, when the same OVA-adsorbed aluminum hydroxide suspension was frozen by placing it on a -80ºC or -20ºC shelf before lyophilization, significant aggregations were detected (Figs. 1D-E). Zapata *et al*. reported that aluminum hydroxide gel could form aggregates ranged from 65 to 160 μm after just one freeze-thaw cycle at -24º C (60). It is thought that the particle coagulation/aggregation is due to the large water crystals formed during the slow freezing process, which bring aluminum hydroxide particles close enough to overcome repulsive forces and cause aggregation, and the original aluminum hydroxide suspension could not

be reproduced upon coagulation (63). By increasing the freezing rate, only smaller ice crystals are formed as a result of a greater rate of nucleation, which are not strong enough to overcome the repulsive forces between particles, and particle aggregation is prevented consequently (63). In the TFF process, a solution or suspension is spread out on a cryogenic substrate to form a thin film in less than one second (cooling rate, ~100 K/s) (70), which may explain why there was not significant aggregation after the OVA-adsorbed aluminum hydroxide was subjected to TFFD (and reconstitution). As mentioned early, it was reported previously that higher cooling/freezing rates help minimize aggregation/agglomeration of vaccines adjuvanted with aluminum salts during freeze-drying (63, 68).

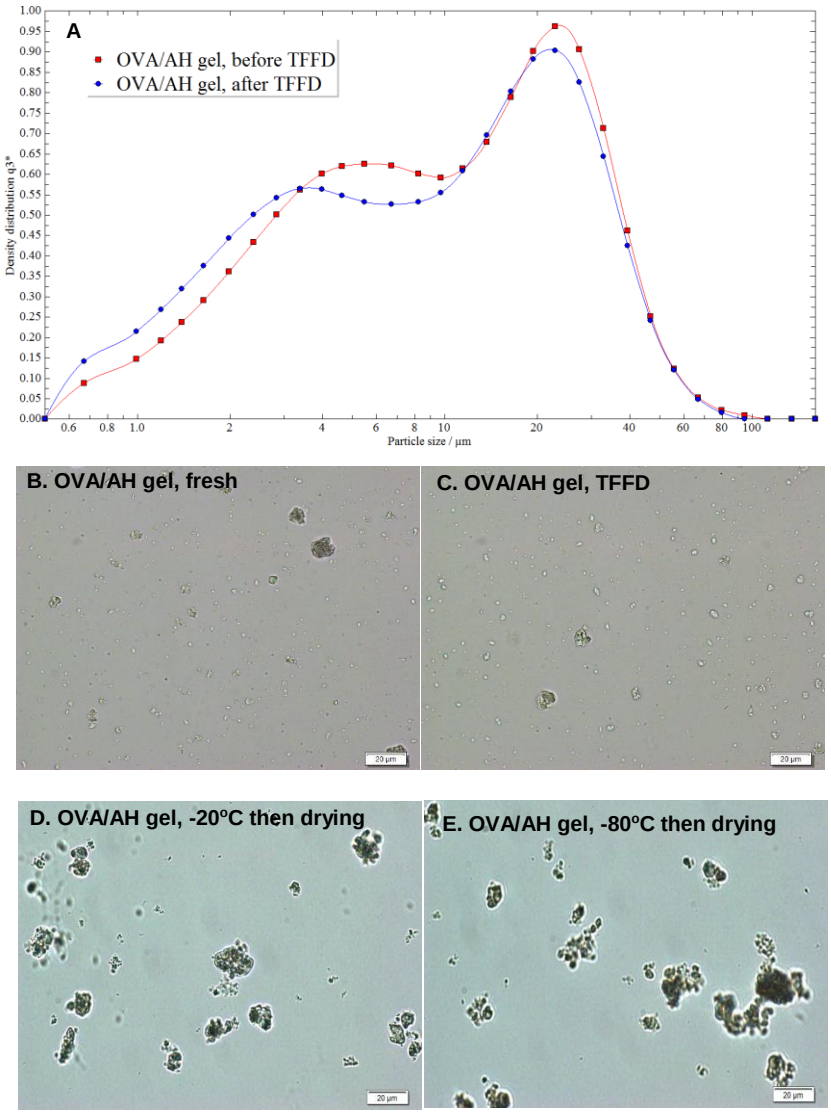


Figure 2.1 TFFD of OVA-adsorbed aluminum hydroxide (OVA-AH gel)

(**A**) Particle size distribution before and after the vaccine was subjected to TFFD and reconstitution. (**B-C**) Representative microscopy images of OVA-AH gel before (B) and after lyophilization and reconstitution (C-E) with 2% trehalose (w/v). In C-E, the method of freezing was TFF, shelf-freezing at -20°C, and shelf-freezing at -80°C, respectively.

2.3.2 Thin-film freeze-drying of OVA-adsorbed aluminum hydroxide in various concentrations of trehalose

Certain sugars, such as trehalose, mannitol, dextran, and sucrose, have been shown to be effective at maintaining protein activity and stabilize aluminum salts in vaccine formulations during freezing process (66, 120, 121). Trehalose forms fragile glass during freezing, resulting in an increase on the viscosity, which limits the mobility of protein molecules or aluminum salt particles, and thus prevents coagulation (120, 122). The formation of glass also resulted in a trehalose-containing phase with maximum concentration that prevents the non-ice concentration or pH-induced aggregation of aluminum salts during freezing (120). The effect of the concentration of trehalose on spray freeze-drying vaccines that contain aluminum hydroxide or aluminum phosphate was previously studied, and it was concluded that 5-20% (w/v) of trehalose was required to successfully spray freeze-dry vaccines adjuvanted with aluminum salts (69). To determine the minimal concentration of trehalose needed to prevent aggregation during TFFD, OVA-adsorbed aluminum hydroxide (AH gel) suspended in various concentrations of trehalose (i.e., 0%, 1%, 2%, 3%, 4%, 5%, w/v) was subjected to TFFD. As shown in Fig. 2A, when the OVA-adsorbed aluminum hydroxide suspension was subjected to TFFD in the absence of trehalose, the mean size of particles after reconstitution was significantly larger than that in the freshly prepared OVA-adsorbed aluminum hydroxide suspension, indicating that a cryoprotectant such as trehalose is needed to successfully convert the OVA-adsorbed aluminum hydroxide into a powder by TFFD. Trehalose at 1% (w/v) was not sufficient (Fig. 2A), but 2% of trehalose was enough to help successfully convert the OVA-adsorbed

aluminum hydroxide into a dry powder following TFFD, without causing particle aggregation (Fig. 2A). Shown in Fig. 2B are the particle size distribution curves of the OVA-adsorbed aluminum hydroxide containing various percentages of trehalose after subjected to TFFD and reconstitution, and the representative images of OVA-adsorbed aluminum hydroxide that were subjected to TFFD with 1%, 2%, and 3% (w/v) of trehalose, respectively, are shown in Fig. 2C. In the present study, trehalose alone was used during the TFFD process. It is expected that other cryoprotectants such as sucrose, glycine and other amino acids, and polymers such as polyvinylpyrrolidone will also help prevent aggregation during the TFFD process. For example, in a previous study, Alum-HBsAg vaccine was suspended in mannitol, glycine, and dextran before spray freeze-drying (63). The concentration of trehalose needed to successfully thin-film freeze-dry OVA-adsorbed aluminum hydroxide was only 2% (w/v) (Figs. 2A-B). Trehalose at concentrations of above 7.5% or above is generally used when spray freeze-dry vaccines adjuvanted with aluminum salts (67-69). Clausi *et al*. actually spray freeze-dried and tray freeze-dried lysozyme adjuvanted with aluminum hydroxide or aluminum phosphate in 2% (w/v) of trehalose (68). The particle size of the lysozyme vaccines increased slightly following freeze-drying and reconstitution, as compared to the untreated lysozyme vaccines (68). Interestingly, in the lysozyme

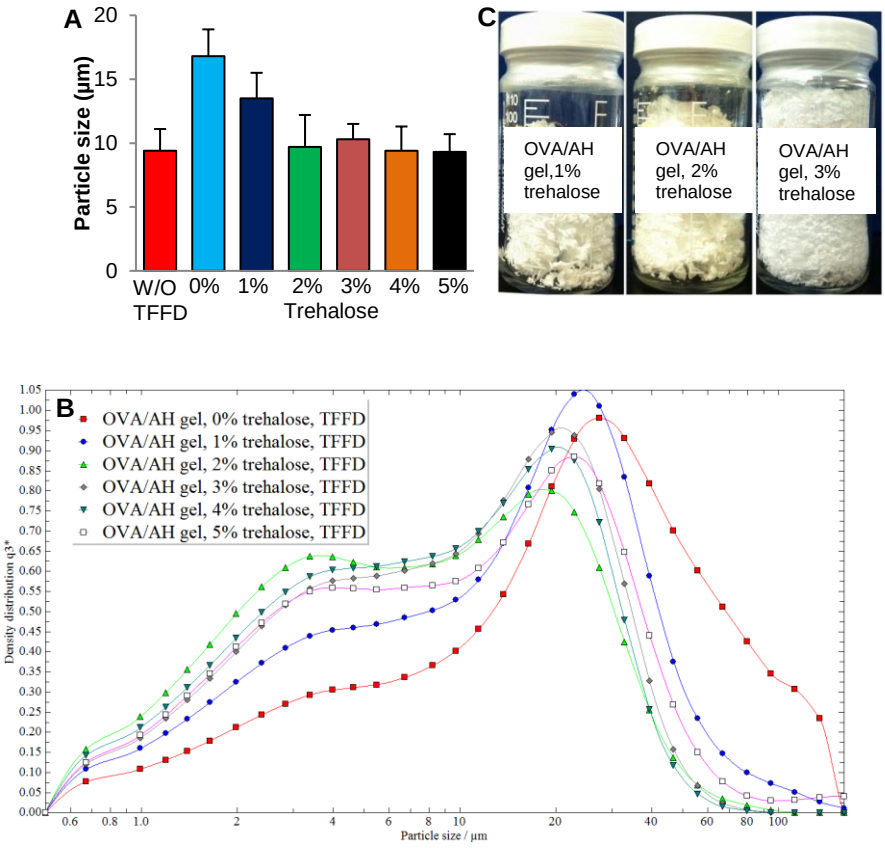


Figure 2.2 TFFD of OVA-adsorbed aluminum hydroxide in various concentrations of trehalose

(A-B). Particle sizes (A) and particle size distribution (B) of OVA-AH gel reconstituted from powders that were thin-film freeze-dried using various concentrations of trehalose (i.e., 0-5%, w/v). **(C).** Representative images of the dried OVA-AH gel powders prepared with 1%, 2%, or 3% (w/v) trehalose, respectively.

vaccines prepared, only 10% of the lysozymes were bound to aluminum salts, and freeze-drying helped increase the percent of lysozymes that adsorbed to aluminum salts (68).

2.3.3 Characterization of the thin-film freeze-dried powder of OVA-adsorbed aluminum hydroxide

To understand the influence of the TFFD process on aluminum hydroxide-adjuvanted vaccines, several studies were conducted to characterize the dried powder of the OVA-adsorbed aluminum hydroxide. The desorption of OVA from the aluminum hydroxide (AH gel) after the OVA-adsorbed aluminum hydroxide was subjected to TFFD was evaluated using SDS-PAGE. It is assumed that the intensity of the OVA band on the SDS-PAGE gel image is inversely correlated to the level of free, unbounded OVA in the OVA-adsorbed aluminum hydroxide preparation (Fig. 3A). At the OVA to Al^{3+} weight ratio of 1:10, almost all OVA (~98.1%) were bound on the aluminum hydroxide (Fig. 3A, NON TFF). After the OVA-adsorbed aluminum hydroxide was subjected to TFFD and reconstitution, the percent of OVA that remained adsorbed on the aluminum hydroxide was estimated to be 91.3% (Fig. 1A, TFF), suggesting that about 7.2% of (loosely) bound OVA protein was desorbed from aluminum hydroxide after the OVA-adsorbed aluminum hydroxide was subjected to TFFD and reconstitution. The binding efficiency of the OVA to aluminum hydroxide before and after it was subjected to TFFD and reconstitution, as determined by measuring the amount of the OVA remained in the supernatant after the vaccine was subjected to centrifugation, was 50.8 ± 13.5% and 62.5 ± 8.6%, respectively ($p = 0.36$), which are lower than that determined using the SDS-PAGE electrophoresis

mentioned above. When the centrifugation method was used, it is assumed that all OVA proteins adsorbed on aluminum hydroxide particles were precipitated after the centrifugation. When SDS-PAGE electrophoresis method was used, it is assumed that all OVA proteins that adsorbed on the aluminum hydroxide particles did not migrate out of the loading wells of the SDS-PAGE gel. The different assumptions may be in part responsible for the different binding efficiency values obtained using those two methods. Nonetheless, it appears that subjected the OVA-adsorbed aluminum hydroxide to TFFD and reconstitution only caused minimum desorption of the OVA from the aluminum hydroxide. Finally, it is noted that in this study, sterile PBS (10 mM, pH 7.4) was used to dissolve OVA and to prepare aluminum hydroxide (AH gel) suspension to minimize OVA denaturing. It was previously reported that pretreatment of aluminum hydroxide with selected concentrations of phosphate ions reduces the positive surface charge, which exists on the aluminum hydroxide at pH 7.4 (113) . The isoelectric point of OVA was reported to be around pH 4.55-4.88 (123). Therefore, if PBS is replaced with other buffers that do not contain phosphate ions, the binding efficiency of the OVA to the aluminum hydroxide particles may increase and the ratio of the OVA to aluminum may need to be adjusted.

Modulated DSC was used to study the thermal properties of the OVA-adsorbed aluminum hydroxide dry powder. The DSC thermogram of the OVA-adsorbed aluminum hydroxide dry powder shows a glass transition temperature (T*g*) of about 120^{o}C (Fig. 3B), indicating that the OVA-adsorbed aluminum hydroxide particles suspended in trehalose solution may have formed a glass after subjected to TFFD (124). The high T*g* value of

~120ºC suggests that the OVA-adsorbed aluminum hydroxide dry powder is highly stable (125, 126).

Shown in Fig. 3C is a representative SEM image of the OVA-adsorbed aluminum hydroxide dry powder. It appears that the OVA-adsorbed aluminum hydroxide particles, which have a rough surface and irregular shape (Fig. 3D), are embedded in the bulk structure of the trehalose (Fig. 3C inset). It is likely that the trehalose surrounding the OVA-adsorbed aluminum hydroxide particles prevented the particles from interacting with one another during the freeze-drying process and prevented their aggregation.

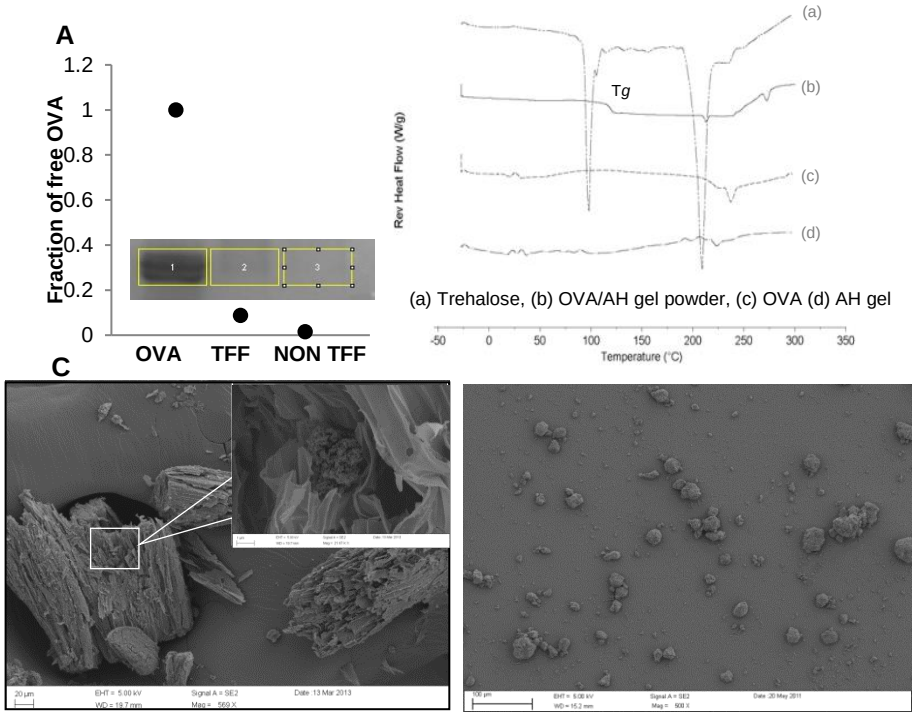


Figure 2.3 Characterization of OVA-adsorbed aluminum hydroxide powder prepared with TFFD

(A). The binding efficiency of OVA to AH gel before and after TFFD (inset, OVA protein band in SDS-PAGE gel). The binding efficiency was measured after the OVA-adsorbed AH gel was subjected to SDS-PAGE electrophoresis. **(B).** DSC curves of OVA-adsorbed AH gel dry powder, OVA, trehalose, and aluminum hydroxide alone. **(C).** A representative SEM image OVA-adsorbed AH gel dry powder. **(D).** A representative SEM image of the freshly prepared OVA-adsorbed AH gel.

2.3.4 The immunogenicity of the OVA-adsorbed aluminum hydroxide after thin-film freeze-drying

To test whether the OVA-adsorbed aluminum hydroxide after subjected to TFFD retained its immunogenicity, the anti-OVA immune responses induced by OVA-adsorbed aluminum hydroxide, freshly prepared or reconstituted from TFFD powder, were evaluated in a mouse model. As shown in Fig. 4, the anti-OVA IgG levels in mice that were immunized with OVA-adsorbed aluminum hydroxide following TFFD and reconstitution were not different from that in mice that were immunized the freshly prepared OVA-adsorbed aluminum hydroxide, regardless of the dose of OVA antigen used (i.e., 5, 10, or 20 μg/mouse/injection). Clearly, the TFFD process not only avoided causing particle aggregation, but also preserved the immunogenicity of the OVA-adsorbed aluminum hydroxide vaccine.

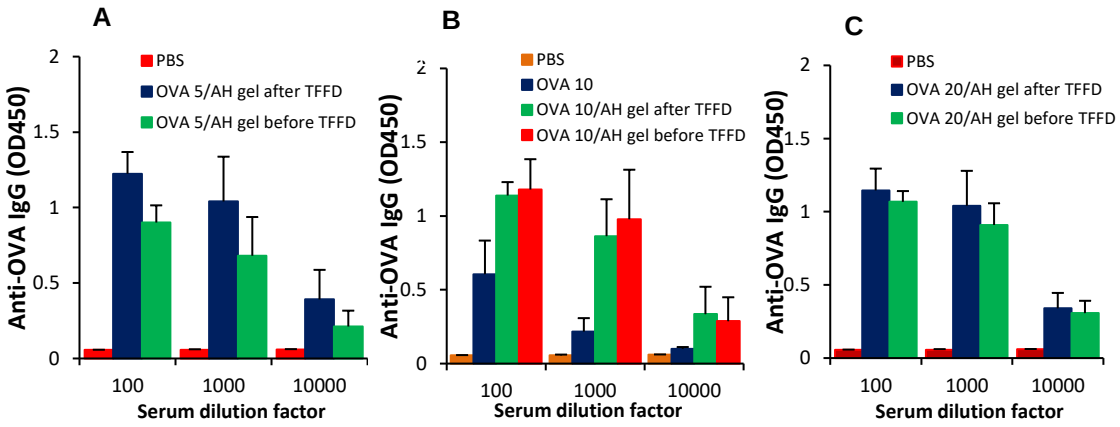


Figure 2.4 Serum anti-OVA IgG levels in mice immunized with OVA-adsorbed aluminum hydroxide, before and after TFFD and reconstitution

Female BALB/c mice (n = 5) were s.c. injected with OVA-adsorbed AH gel, before or after it was subjected to TFFD and reconstitution, on days 0, 14 and 28 with 5 µg (**A**), 10 µg (**B**), or 20 µg (**C**) of OVA per mouse. The ratio of OVA to aluminum was 1 to 10. Sterile PBS and OVA alone (10 µg) in PBS were used as controls. Total anti-OVA IgG levels in serum samples were measured 16 days after the third dose. Data shown are mean ± S.D.

2.3.5 Thin-film freeze-drying of OVA-adsorbed aluminum phosphate and OVA-adsorbed Alhydrogel®

Both aluminum hydroxide and aluminum phosphate are commonly used in human vaccines. Therefore, the feasibility of using TFFD to dry a protein antigen adjuvanted with aluminum phosphate into a powder was tested using OVA as a model antigen. Moreover, in the above studies, the aluminum hydroxide suspension (AH gel) was prepared in our own laboratories by dispersing dried aluminum hydroxide gel (USP grade) in water. Alhydrogel® (2%, w/v) is a commercially available aluminum hydroxide wet gel suspended in normal saline. Therefore, the feasibility of drying OVA-adsorbed Alhydrogel® using TFFD was also tested. Both OVA-adsorbed aluminum phosphate and OVA-adsorbed Alhydrogel® were successfully converted into powders using TFFD. Both dried samples appeared as light white-colored powder and were easily reconstituted in water with no or minimum agitation. The particle size distribution profiles of the OVA-adsorbed aluminum phosphate before and after subjected to TFFD and reconstitution are shown in Fig. 5A, and the particle size distribution profiles of the OVA-adsorbed Alhydrogel® before and after subjected to TFFD and reconstitution are shown in Fig. 5B. Therefore, TFFD can be used to convert vaccines adjuvanted with aluminum phosphate or with the commercially available Alhydrogel® into dry powder without causing significant aggregation.

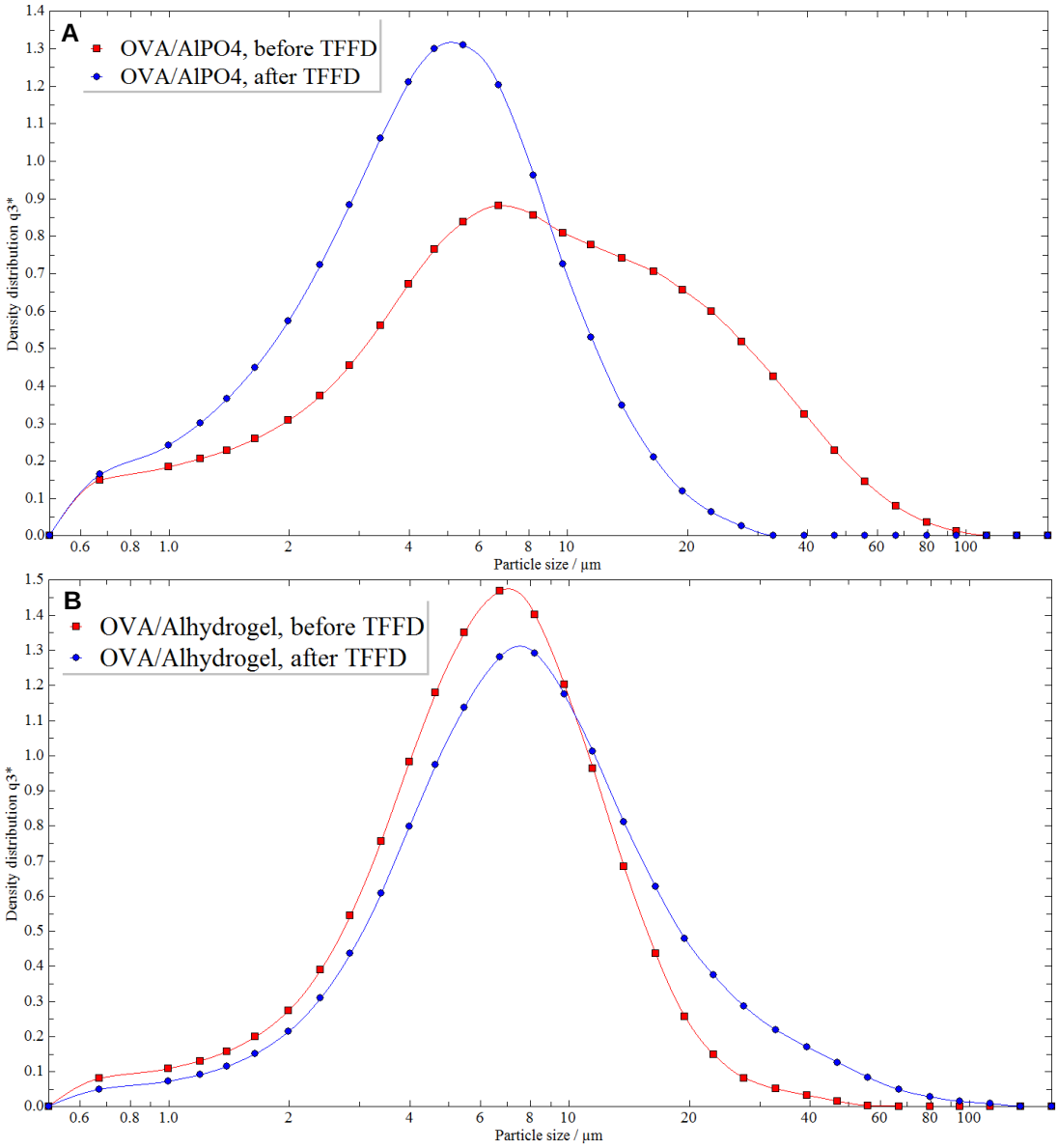


Figure 2.5 TFFD of OVA adjuvanted with aluminum phosphate or Alhydrogel®

Shown are representative particle size distribution curves of OVA-adsorbed aluminum phosphate (A) and OVA-adsorbed Alhydrogel® (B) before and after they were subjected to TFFD and reconstitution.

2.3.6 Thin-film freeze-drying of a commercial veterinary tetanus toxoid vaccine, a human hepatitis B vaccine, and a human papillomavirus vaccine

In order to further validate the applicability of the TFFD in drying vaccines adjuvanted with aluminum salts, the tetanus toxoid concentrated, adjuvanted detoxified toxin, a veterinary TT vaccine, Engerix-B, a human hepatitis B vaccine, and Gardasil, a human papillomavirus vaccine, were subjected to TFFD.

The TT vaccine was diluted, and trehalose was added to a final concentration of 2% (w/v) before the vaccine was subjected to TFFD. The particle size distribution profiles of the TT vaccine before and after being subjected to TFFD and reconstitution are shown in Fig. 6A. The mean diameter of the particles in the original vaccine was 23.1 ± 2.1 μm, and the mean particle size the TT vaccine reconstituted from the dried powder was 18.4 ± 0.2 μm. Preliminary data showed that subjected undiluted TT vaccine in the presence of 2% (w/v) of trehalose to TFFD and reconstitution did not cause particle aggregation as well (Thakkar & Cui, unpublished data). Clearly, subjecting the TT vaccine to TFFD and reconstitution did not cause significant aggregation.

To investigate whether the TFFD process significantly altered the structure of the tetanus toxoid protein, the intrinsic fluorescence spectra of the TT vaccine before and after it was subjected to TFFD were acquired and compared. As shown in Fig. 6B, the fluorescence spectrum of the TT vaccine after it was subjected to TFFD and reconstitution only shifted slightly right (about 20 nm) when compared to the freshly diluted TT vaccine. In addition, the fluorescence intensity of the TT vaccine following TFFD and reconstitution was also relatively lower. Freeze-drying is known to perturb the structure of proteins at any

stage of the process, including freezing, drying, and reconstitution (67, 127, 128). The TFFD may have slightly altered the structure of the detoxified tetanus toxoid. However, it is unclear how the TFFD increased the polarity of the environment surrounding the tryptophan residues in the detoxified tetanus toxoid to induce a slight right shift in the spectra. When the immunogenicity of the TT vaccine before and after it was subjected to TFFD and reconstitution was evaluated and compared in a mouse model, the anti-tetanus toxin IgG levels determined using toxoid-ELISA in all the immunized groups were not significantly different (Fig. 6C). For anti-TT antibody responses, specific IgG levels measured using the toxoid-ELISA do not correlate well with tetanus toxin-neutralizing activity determined using *in vivo* toxin neutralization assay in mice or guinea pigs (117). However, it was shown that the ToBI-test is a reliable and precise alternative to the *in vivo* toxin neutralization assay, and there is a high degree of correlation between the ToBI-test and *in vivo* toxin neutralization assay (117). Therefore, the sera from mice immunized with the TT vaccine before and after it was subjected to TFFD and reconstitution were evaluated using the ToBI-test and compared. As shown in Fig. 6D, subjecting the TT vaccine to TFFD and reconstitution did not significantly affect the ToBI-test results. Although an *in vivo* toxin neutralization assay may ultimately have to be carried out to assess the effect of the TFFD on the toxin-neutralizing activity of the antiserum induced by the vaccine, data in Figs. 6C-D strongly indicated that the immunogenicity of the vaccine was not significantly decreased after it was subjected to TFFD and reconstitution.

Engerix-B vaccine is a human hepatitis B vaccine, which contains human HBsAg adjuvanted with aluminum hydroxide (HBsAg to aluminum ratio, 1:25, w/w). To further

test the applicability of the TFFD process in drying commercially available vaccines adjuvanted with aluminum salts, trehalose powder was added directly into the Engerix-B vaccine to a final concentration of 2% (w/v) without further dilution, and the formulation was then subjected to TFFD. Shown in Fig. 7A are representative particle size distribution profiles of the Engerix-B vaccine before and after it was subjected to TFFD and reconstitution. The particle size of the Engerix-B after it was subjected to TFFD and reconstitution was 3.29 ± 0.15 μm, and particle size of the fresh Engerix-B vaccine was 5.64 ± 0.01 μm. Gardasil is a human papillomavirus qaudrivalent vaccine that contains L1 proteins from HPV 6, 11, 16, and 18 adjuvanted with amorphous aluminum hydrooxyphosphate sulfate (total L1 proteins to aluminum ratio, 1:1.875, w/w). It was diluted 10-fold (with a final trehalose concentration of 2% (w/v)) and then subjected to TFFD. Shown in Fig. 7B are representative particle size distribution curves of the Gardasil before and after it was subjected to TFFD and reconstitution. Clearly, subjecting human Engerix-B vaccine or Gardasil vaccine to TFFD and reconstitution did not cause any significant aggregation. Therefore, it is likely that the TFFD method can be used as a platform technology to convert vaccines that contain aluminum salts into dry powder.

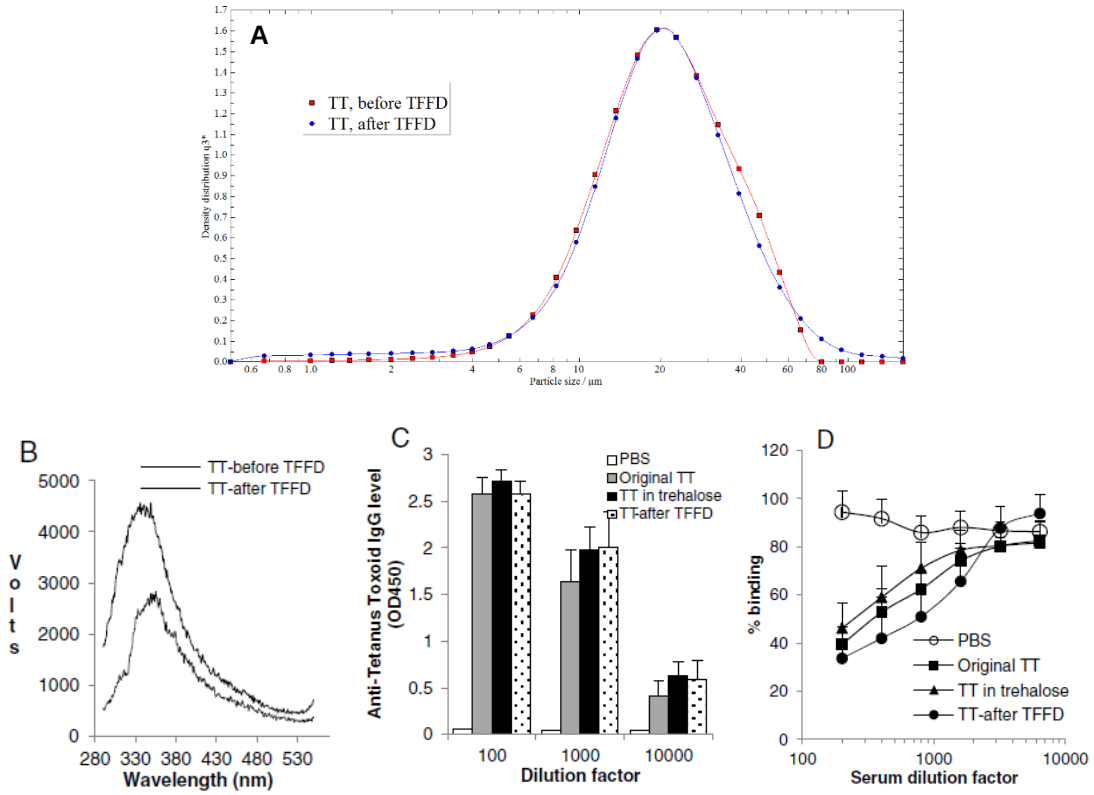


Figure 2.6 TFFD of tetanus toxoid vaccine

(**A**) Representative particle size distribution curves of TT vaccine before and after it was subjected to TFFD and reconstitution. (**B**) Intrinsic tryptophan fluorescence spectra of TT vaccine before and after TFFD and reconstitution (**C**) Anti-tetanus toxin IgG levels in serum samples of mice immunized with TT vaccine before and after TFFD and reconstitution. (**D**) Inhibition of the binding of tetanus toxoid (to polyclonal horse anti-tetanus antiserum by the antisera from immunized mice). Female BALB/c mice (n = 5) were s.c. injected with TT vaccine, before or after TFFD and reconstitution, on days 0, 14 and 28 with 3.75 Lf of tetanus toxoid per mouse per injection. Sterile PBS and original TT vaccine diluted in sterile PBS (original TT) or 2% trehalose (TT in trehalose) were used as controls. Mice were bled 16 days after the third dose.

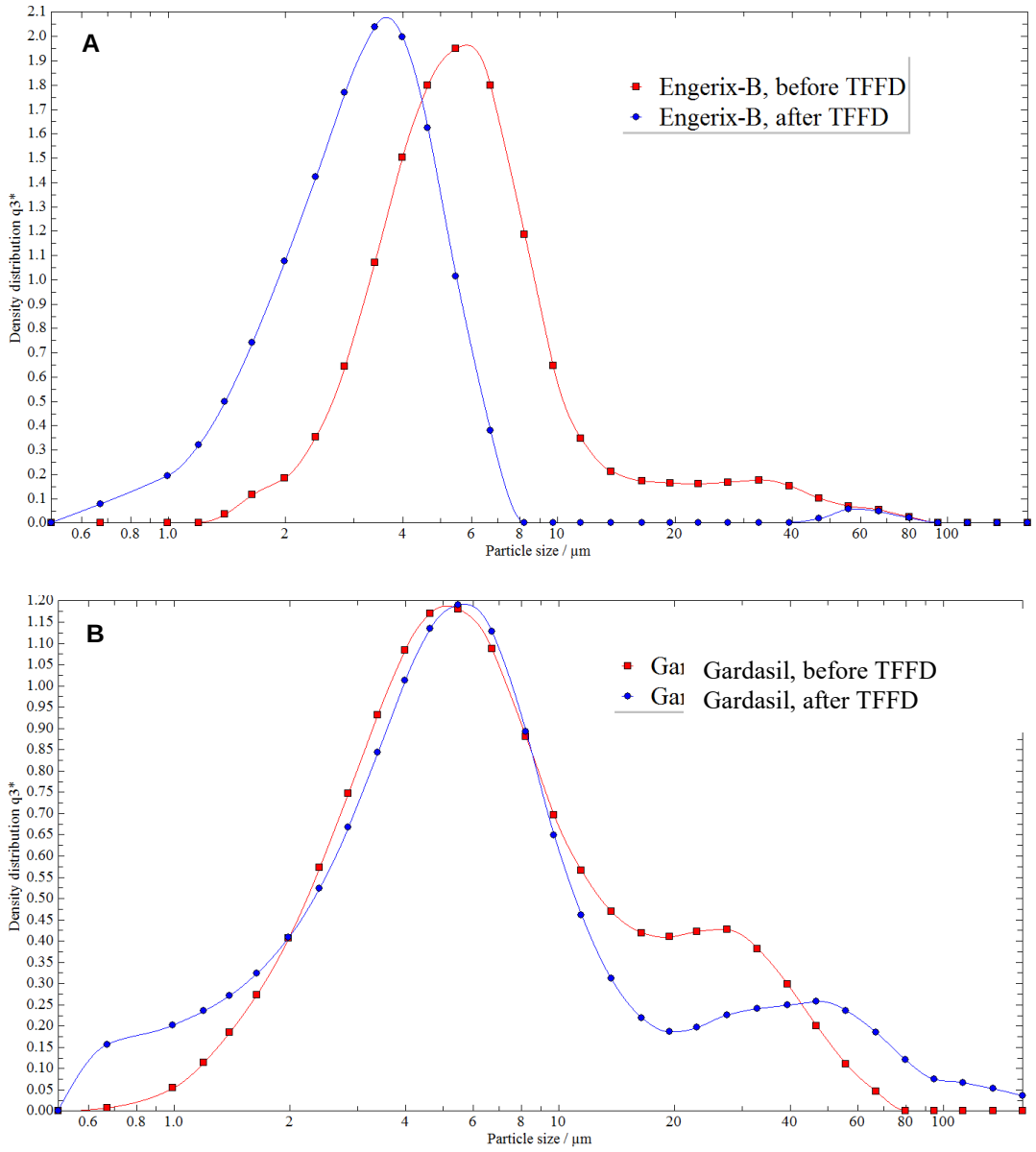


Figure 2.7 TFFD of Engerix-B and Gardasil

Shown are representative particle size distribution curves of Engerix-B (A) or Gardasil (B) vaccines before and after they were subjected to TFFD and reconstitution. Engerix-B was subjected to TFFD in 2% of trehalose without further dilution. Gardasil was diluted 10-time and then subjected to TFFD in 2% of trehalose.

2.3.7 Stability of vaccine dry powder after repeated freezing-and-thawing

To test whether vaccines that are converted to dry powder using TFFD are still sensitive to inadvertent freezing (and thawing), dried powders of TT vaccine, Engerix-B vaccine, and OVA-adjuvanted Alhydrogel® (OVA/Alhydrogel®) were subjected to three cycles of freezing-and-thawing and then reconstituted. As a control, fresh vaccines that were not subjected to TFFD were also subjected to the same freezing–and-thawing cycles. As shown in Fig. 8, repeated freezing-and-thawing of all three vaccines in suspension caused significant aggregations, whereas subjecting the dried powders of the same vaccines to the same freezing-and-thawing cycles did not cause any significant aggregation. It appears that unlike liquid vaccines that are adjuvanted with aluminum salts, the same vaccines that are converted to dry powders using the TFFD were not sensitive to freezing conditions anymore. Therefore, converting a vaccine that contains an aluminum salt as adjuvant from liquid to dry powder using TFFD is expected to help avoid the loss of vaccines resulted from unintentionally exposing the vaccine to freezing temperatures during shipment or storage (46, 110). Experiments to evaluate and compare the immune responses induced by thin-film freeze-dried vaccine powder before and after it is subjected to multiple cycles of freezing-and-thawing (and reconstitution) are currently underway.

As mentioned earlier, vaccines adjuvanted with aluminum salts were successfully converted to dry powder by various methods including spray drying (63), spray freeze-drying (63, 67-69), and standard tray freeze-drying (67-69, 129). However, data in the literature do not show that those methods truly preserved the particle size and the

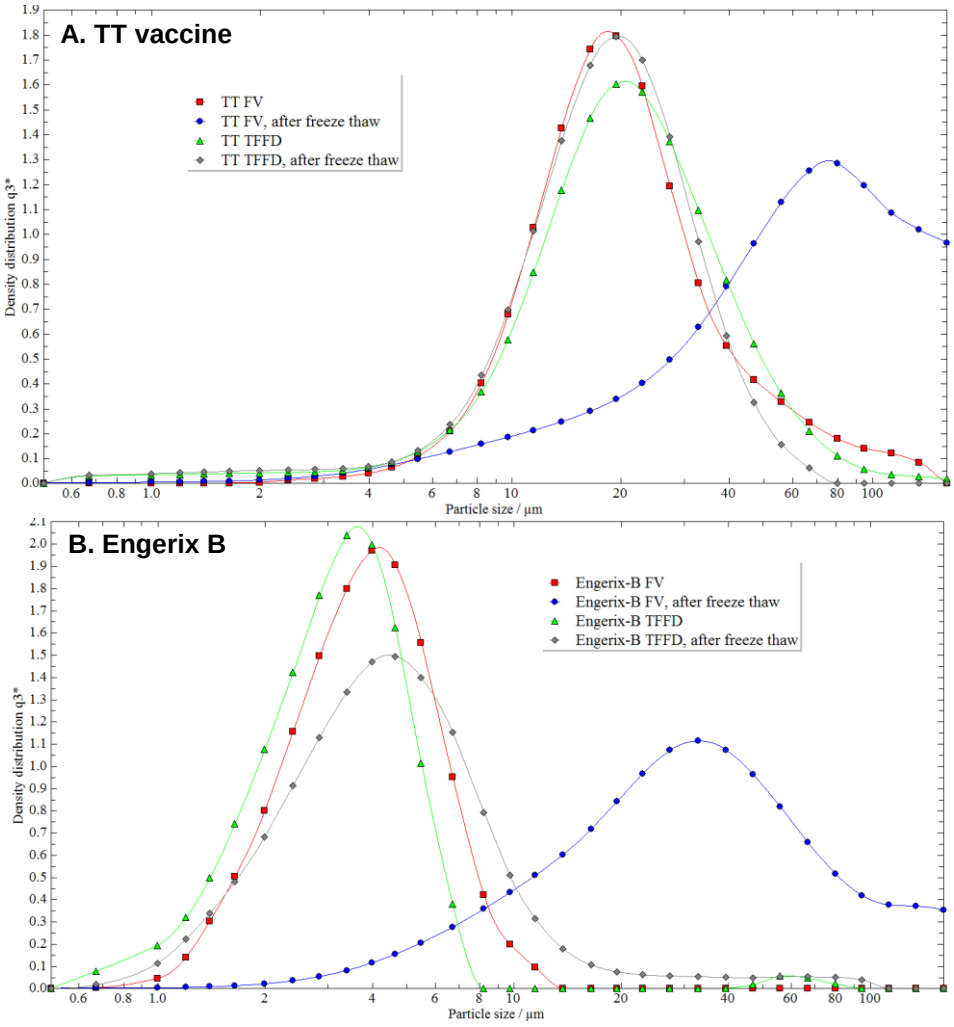


Figure 2.8 Freezing-and-thawing of vaccine dry powder

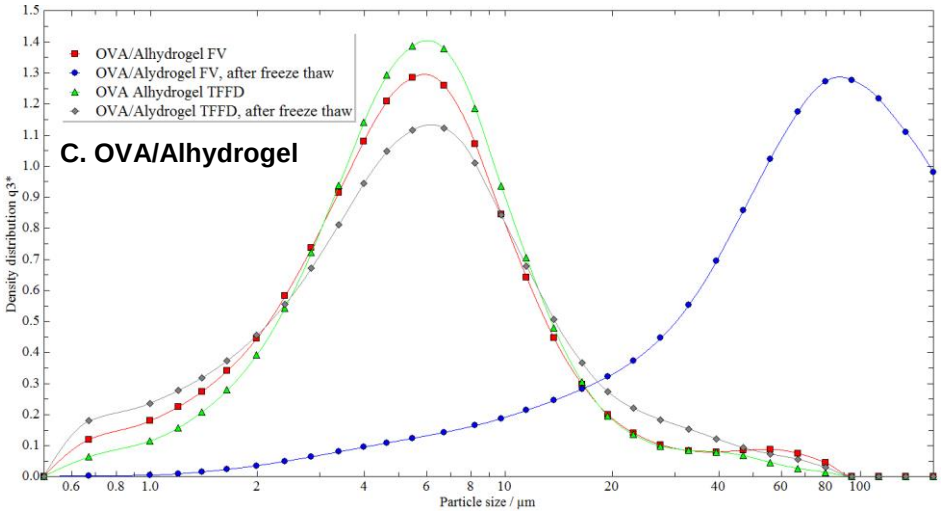


Figure 2.8 (continued)

Shown are representative particle size distribution curves of TT vaccine (A), Engerix B (B), and OVA adjuvanted with Alhydrogel (OVA/Alhydrogel) (C), before and after the thin-film freeze-dried powders were subjected to 3 cycles of freezing-and-thawing and then reconstitution (i.e., TFFD vs. TFFD, after freeze thaw). As a control, the particle size distribution curve of the respective fresh vaccines (i.e., FV) and the fresh vaccines after subjected to 3 cycles of freezing-and-thawing (i.e., FV, after freeze thaw) are also included.

immunogenicity of the vaccines (63, 67-69). They are rather methods of preparing immunologically-active aluminum salt-adjuvanted dry vaccines, albeit with reduced immunogenicity (69). The thin-film freeze-drying method used in the present study represents a method that converts vaccines adjuvanted with aluminum salts from a liquid suspension to a dry powder without causing particle aggregation or damage to the immunogenicity of the vaccines. In TFF process, liquid droplets fall from a given height above a cryogenically cooled metal surface (70). Upon impact, the droplets spread out into thin films of 100-400 μm that froze on a time scale of 70-1,000 ms, which corresponding to a cooling rate of about 100 K/s (70, 130-135). The rapid cooling/freezing explains why the particle size (distribution) and the immunogenicity of vaccines adjuvanted with aluminum salts were not significantly changed after they are subjected to TFFD and reconstitution, as it was reported that fast freezing can minimize particle coagulation and thus maximizing the immunogenicity of vaccines adjuvanted with aluminum salts (63, 68, 69). However, in the previously reported spray freeze-drying method, when atomized vaccine droplets were dropped above liquid nitrogen, they traveled through the cold gas above the cryogenic liquid nitrogen and completely froze at an estimated cooling rate of about 10^6 K/s after contacting the liquid nitrogen (136). In other words, the cooling rate during spray freezing is in theory much higher than during the TFFD process. It is thus surprising that in previous reports, aluminum salt-adjuvanted vaccines after subjected to spray freeze-drying and reconstitution did not maintain their particle size and/or immunogenicity (63, 67-69).

Besides cooling rate, low aluminum salt concentration and high excipient levels are also indicated as two important parameters that help minimize vaccine aggregation and maximize the immunogenicity of vaccines (63, 68). In the present study, the concentrations of the aluminum hydroxide in the vaccine formulations we prepared ranged from 0.09% to ~1% (w/v). The concentration of aluminum in the Engerix-B was ~0.5 mg/ml, 0.045 mg/ml in the diluted Gardasil (the concentration of aluminum in the TT vaccine is unknown). It was found that trehalose as a single excipient at 2% (w/v) was sufficient to help maintain the particle size (distribution) and immunogenicity of the vaccines during the TFFD and reconstitution process. In previous spray freeze-drying studies, the aluminum hydroxide concentration was 0.2% (w/v) (67-69) or 0.6% (w/v) (63). Aluminum hydroxide at a final concentration of 3% (w/v) was also tested, but the immunogenicity of the resultant powder after reconstitution was significantly decreased (i.e., 10-fold lower than that of the original liquid vaccine) (63). The concentration of trehalose as a single excipient was 7.5% (w/v) or above (67-69), and in Maa *et al*.'s study, the excipient was comprised of mannitol, dextran, and glycine (63). Yet, the particle size and/or immunogenicity of the vaccines changed after subjected to spray freeze-drying and reconstitution (63, 67-69).

Therefore, fast cooling rates, low aluminum salt concentrations, and high excipient levels are critical factors/parameters in minimizing vaccine aggregation and maximizing the immunogenicity of vaccines during freeze-drying. However, other factor(s) may also play an important role in maintaining the particle size and immunogenicity of aluminum salt-adjuvanted vaccines during freeze-drying and reconstitution. For example, the

antigens used in the freeze-drying process may be critical. Lysozyme, alkaline phosphatase, BoNT/E, BoNT/C, and HBsAg were used previously in spray freeze-drying studies (63, 67-69). In the present study, OVA and tetanus toxoid (in a veterinary TT vaccine) were used. The aluminum hydroxide-adjuvanted HBsAg, Engerix-B, and the aluminum hydrooxyphosphate sulfate-adjuvanted HPV L1 proteins, Gardasil, were also subjected to TFFD, but the immunogenicity of the vaccines after subjected to TFFD and reconstitution was not tested yet. More importantly, however, it is known that during spray freeze-drying, the large gas-liquid interface in the spraying step causes protein aggregations (136-138). The TFF process freezes liquid droplets dropped on the cryogenically cooled metal surface in relatively lower cooling rates, but the much smaller area of the air-liquid interface of the falling droplets and the spread film, in comparison to the atomized droplets in spray freeze-drying (70), may have contributed to the ability of the TFFD process to convert liquid aluminum salt-adjuvanted vaccine to dried powder without causing significant change in the particle size and immunogenicity of the vaccines.

2.4 CONCLUSION

Vaccines that are adjuvanted with aluminum salts can be successfully converted from liquid suspension into dry powder by thin-film freeze-drying using a relatively low concentration of trehalose (2%, w/v) as an excipient, without causing particle aggregation or decrease in the immunogenicity of the vaccines. In addition, the dry vaccine powder did not aggregate after repeated freezing-and-thawing. It is expected that this thin-film freeze-drying method can be used to formulate new vaccines, or to reformulate existing vaccines, that are adjuvanted with aluminum salts into dry vaccine powder.

Chapter Three

The Immunogenicity of thin-film freeze-dried, aluminum salt-adjuvanted vaccine when exposed to different temperatures[2]

3.1 INTRODUCTION

Some insoluble aluminum salts, e.g. aluminum oxyhydroxide and aluminum hydroxyphosphate, have been widely used as human vaccine adjuvants for decades, and many currently licensed and commercially available vaccines, including those for diphtheria-tetanus-pertussis, hepatitis A and B, pneumococcal disease, anthrax, and rabies, contain aluminum salts as adjuvants (139-141). The primary particles of aluminum oxyhydroxide and aluminum hydroxyphosphate are in the nanometer range. However, when dispersed in an aqueous solution, these particles aggregate to form large microparticles (e.g. 1-20 μm) (141, 142). Thus, a vaccine that is prepared by binding an antigen onto an aluminum salt is physically a liquid suspension of aluminum salt particles with antigens adsorbed on them.

Protein antigens adsorbed on aluminum salt adjuvants in liquid suspension are generally too fragile to be stable in ambient temperatures. Also, exposing the liquid suspension of aluminum salt-adjuvanted vaccines to slow freezing causes irreversible aggregation of the aluminum salt particles that damages the vaccines (e.g. permanent loss

[2] This chapter is based on "**Sachin G. Thakkar**, Tinashe B Ruwona, Robert O. Williams III, Zhengrong Cui, ***Human Vaccines & Immunotherapeutics****, January 2017, Vol. 13, No. 4, 936–946*

in potency and/or effectiveness) (60, 143-147). Because of these reasons, aluminum salt-adjuvanted vaccines must be maintained in cold-chain (i.e. 2-8^{o} C) during production, handling, transport, storage, and use (148). Unfortunately, published reports and field evidence demonstrate that inadvertent freezing of vaccines during transport and storage in cold-chain is a commonplace, and not resource limited (110, 146, 149-153). This in effect causes the widespread delivery of vaccines with compromised potency, which could result in unintentional administration of suboptimal vaccines to patients (145, 154-158), and/or costly waste of global vaccine supplies (48, 150), Meanwhile, as the costs and/or logistical constraints of vaccine delivery associated with the cold-chain requirements significantly obstructs global vaccine access, there is increasing interest in novel approaches to vaccine stability management such as controlled temperature chain (CTC) storage (45, 46). CTC allows vaccines to be managed in temperatures outside of the traditional cold-chain for a limited period of time, typically a single excursion into ambient temperature not exceeding 40° C for the duration of a specific number of days prior to administration (47). For example, in 2012, MenAfriVac^{TM} became the first to be prequalified by the World Health Organization (WHO) to receive regulatory approval during mass vaccination that allowed vaccine storage at or below 40° C for up to 4 days (45, 48, 49). It is estimated that by using CTC, the costs of using cold-chain and the associated logistics can be reduced by 50% (48).

Thin-film freeze-drying (TFFD) is a rapid freezing technology originally studied to enhance the solubility of poorly water-soluble compounds, and was recently used to prepare stable submicron protein particles (70, 111). In the process of TFFD, droplets of drug formulation are rapidly frozen upon impact with a cryogenically-cooled substrate to

form thin films in less than a second. These thin films are then lyophilized to remove solvent in the formulation (111). Previously, we reported that vaccines adjuvanted with aluminum salts can be successfully converted from liquid suspension to dry powder by TFFD, without causing particle aggregation or decreasing in immunogenicity following reconstitution (159). In an effort to test whether converting vaccines adjuvanted with aluminum salts from liquid suspension to solid dry powder can afford managing the vaccines in CTC, here we tested the stability of a thin-film freeze-dried, aluminum oxyhydroxide-adjuvanted vaccine powder. The stability of the dry vaccine powder was evaluated in temperatures as high as 40° C for up to 6 months by measuring the particle size distribution and the immunogenicity of the vaccine powder upon reconstitution. Moreover, as the risk of unintentional exposure of vaccines to freezing temperatures remains regardless whether the vaccines are managed in cold-chain or CTC, or by any other approaches, we also tested the immunogenicity of a thin-film freeze-dried, aluminum oxyhydroxide-adjuvanted vaccine powder after it was subjected to repeated slow freezing-and-thawing cycles (and then reconstitution) in a mouse model. Data from our previous study showed that subjecting thin-film freeze-dried, aluminum salt-adjuvanted vaccines to repeated slow freezing-and-thawing cycles does not cause particle aggregation upon reconstitution (159).

Commercial vaccines usually contain additional excipients such as buffer, stabilizer(s), and preservative(s) that are unique and often proprietary to each vaccine and could potentially protect the vaccines from freezing or heat stress to a certain degree. Therefore, in the present study, we prepared a model vaccine by adsorbing ovalbumin

(OVA) as a model antigen onto Alhydrogel®, i.e. aluminum oxyhydroxide (2%, w/v), and used it for the immunogenicity evaluations. It is known that freezing or heating OVA denatures the protein (160, 161), and denatured OVA is less immunogenic than native OVA and has different immunogenic epitopes (162, 163). There are also reports that certain excipients such as high concentrations of phosphate (e.g. 40 or 100 mM) and histidine can help improve the thermal stability of vaccine, and polyols such as propylene glycol, polyethylene glycol 300, and glycerol can help make vaccine insensitive to freezing (164-166). In this study, the OVA-adsorbed Alhydrogel® liquid vaccine was prepared in the absence of those excipients, making it ideal, in our opinion, for the present study. The OVA-adsorbed Alhydrogel® dry powder was prepared by TFFD using OVA-adsorbed Alhydrogel® liquid vaccine containing 2% (w/v) trehalose (159).

3.2 MATERIALS AND METHODS

3.2.1 Preparation of OVA-adsorbed Alhydrogel® vaccine dry powder

The OVA-Alhydrogel® vaccine was prepared by adding 25 mL of Alhydrogel® (2% w/v, or 10 mg/mL aluminum, manufactured by Brenntag, and supplied by InvivoGen, San Diego, CA) into a 50 mL tube followed by the addition of 25 mL of an OVA solution (1 mg/mL in phosphate-buffered saline (PBS), pH 7.4, 10 mM, Sigma-Aldrich, St. Louis, MO) and 1 g of trehalose (as a cryoprotectant) (Sigma-Aldrich) to obtain a final formulation with 2% (w/v) of trehalose, ~1% (w/v) of Alhydrogel®, and 0.5 mg/mL of OVA, with a low 5 mM concentration of PBS. The size and size distribution of the OVA-adsorbed Alhydrogel® in suspension was determined using a Sympatec Helos laser diffraction instrument equipped with an R3 lens (Sympatec GmbH, Germany). The vaccine suspension was converted into a dry powder using our previously reported thin-film freeze-drying method (70, 111, 159). The powder was dried using a VirTis AdVantage Bench Top Lyophilizer (The VirTis Company, Inc. Gardiner, NY). Lyophilization was performed over 72 h at pressures less than 200 mTorr, while the shelf temperature was gradually ramped up from −40° C to 26° C. After lyophilization, the solid dry vaccine powder was transferred into a sealed container and stored in a desiccator (112). The moisture content in the dried powder was determined using a Karl Fisher Titrator Aquapal III from CSC Scientific Company (Fairfax, VA).

To test the feasibility of preparing dry vaccine powder by thin-film freeze-drying in a single vial, silanized glass vials were used to provide the cryogenic surface for rapidly

freezing the liquid vaccine. Glass vials were submerged into liquid nitrogen to make the cryogenic surface, with the mouth and neck of the vials remaining in the air (not submerged). Using a pipette or syringe, the OVA/Alhydrogel® vaccine liquid suspension with 2% of trehalose (w/v) (0.25-0.5 mL) was added drop wise into the glass vials. Thin films of vaccine were quickly formed by ultra-rapid freezing on the inner surface of the vial, which were then lyophilized as mentioned above. After lyophilization, the glass vials were quickly crimped, transferred to a sealed container, and stored in a desiccator in room temperature before further use. As a control, OVA/Alhydrogel® vaccine liquid suspension in a glass vial was placed on the shelf on the lyophilizer. The shelf temperature was gradually decreased from room temperature to -40° C, and then ramped from −40° C to 26° C over 72 h at pressures of less than 200 mTorr. To test whether the above mentioned freeze-drying method significantly affected the particle size distribution of the vaccine, the lyophilized dry vaccine powder was reconstituted using water. The particle size and particle size distribution were determined using a Sympatec Helos laser diffraction instrument. As controls, the particle size distribution profiles of freshly prepared OVA/Alhyrogel® vaccine and the same OVA/Alhydrogel® vaccine that was subjected to TFFD using the previously reported method and then reconstitution were also evaluated (159).

3.2.2 Stability study

The OVA/Alhydrogel® dry powder was crimp-sealed with aluminum seals over rubber lids in silanized glass vials, which were then stored in desiccators placed in room

temperature (i.e. 22-24° C) or in incubators (30° C or 40° C). As a control, OVA/Alhydrogel® liquid suspension that was crimp-sealed with aluminum seals over rubber lids in silanized glass vials was also stored at 4° C, room temperature, 30° C, and 40° C. At various time points (i.e. 1, 3, or 6 months later), the particle size and size distribution of the vaccine, in liquid suspension or reconstituted from dry powder, were determined using a Sympatec Helos laser diffraction instrument. The immunogenicity of the vaccine was evaluated in a mouse model.

3.2.3 Chemical stability of the OVA protein desorbed from OVA/Alhydrogel®

SDS-PAGE electrophoresis was used to evaluate the chemical integrity of the OVA desorbed from Alhydrogel® after storage as mentioned above. Briefly, OVA/Alhydrogel® dry vaccine powder containing 1 mg of OVA was reconstituted and incubated in the presence of sodium citrate (Sigma-Aldrich) to a final concentration of 10% (w/v) at 37° C overnight. The samples were then centrifuged to collect the supernatant. Control samples included reconstituted OVA-adsorbed Alhydrogel® incubated similarly but in the absence of sodium citrate, freshly prepared OVA/Alhydrogel®, and OVA alone. Samples were mixed with a Laemmli sample buffer (Sigma-Aldrich) before applied to 7.5% Mini-PROTEAN® TGX™ precast polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA). Precision plus™ protein standards (Bio-Rad) were also run along with the samples at 130 V for 1 h. The gels were then stained in a Bio-Safe™ Coomassie blue staining solution and scanned using a Kodak Image Station 440CF (Rochester, NY).

3.2.4 Repeated freezing-and-thawing studies

Thin-film freeze-dried OVA/Alhydrogel® powder was placed at -20° C for 8 h and then thawed at 4° C for 16 h for three cycles. After the third cycle, the dry powder was reconstituted to (i) measure its particle size and size distribution using a Sympatec Helos laser diffraction instrument and (ii) evaluate its immunogenicity in a mouse model. As a control, OVA/Alhydrogel® vaccine in liquid suspension was also subjected to 3 cycles of freezing-and-thawing, and its particle size distribution and immunogenicity were evaluated.

3.2.5 Animal studies

All animal studies were conducted following the U.S. National Research Council Guide for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee at The University of Texas at Austin approved the animal protocol. Female BALB/c mice, 6-8 weeks of age, were from Charles River Laboratories, Inc. (Wilmington, MA). Mice (n = 5) were subcutaneously (s.c.) injected with OVA/Alhydrogel®, freshly prepared, after storage, or after freezing-and-thawing cycles. Mice were immunized on days 0, 14, and 28, and the dose of OVA was 5 μg per mouse. As controls, mice were s.c. injected with sterile PBS or OVA alone in PBS. Sixteen days after the third dose, mice were bled, and the anti-OVA IgG levels in serum samples were determined using enzyme-linked immunosorbent assay (ELISA) (116). Briefly, EIA/RIA flat bottom, NUNC Maxisorp, 96-well plate (Thermo Fisher) were coated with 1 ng/μl of OVA solution in carbonate buffer (0.1 M, pH 9.0) overnight at 4° C. Plates were blocked with horse serum

for one hour before adding the blood serum. Horse radish peroxidase (HRP)-labeled goat anti-mouse immunoglobulin (IgG, 5000-fold dilution, Southern Biotechnology Inc., Birmingham, AL) was added into the plates, and the presence of bound antibody was detected in the presence of 3,3′,5,5′-tetramethylbenzidine solution (TMB, Sigma–Aldrich). The absorbance was read at 450 nm.

3.2.6 Statistics

Statistical analyses were conducted using analysis of variance followed by Fischer's protected least significant difference procedure. A p-value of < 0.05 (two-tail) was considered statistically significant.

3.3 RESULTS AND DISCUSSION

Previously, we reported that vaccines adjuvanted with an insoluble aluminum salt, e.g. aluminum oxyhydroxide, aluminum hydroxyphosphate, or amorphous aluminum hydroxyphosphate sulfate, can be converted from liquid suspension to dry powder without causing particle aggregation or decreasing in immunogenicity following reconstitution (159). In the present study, we tested whether an aluminum salt-adjuvanted vaccine powder prepared by TFFD could be a potential candidate for CTC. CTC allows a single excursion into ambient temperature not exceeding 40° C for the duration of a specific number of days prior to administration. Therefore, we evaluated the immunogenicity of a model vaccine prepared by adsorbing OVA as a model antigen onto Alhydrogel® after it was converted to a dry powder by TFFD in the presence of 2% (w/v) of trehalose and then stored in temperatures as high of 40° C for up to 6 months. OVA was adsorbed onto Alhydrogel® at an OVA to aluminum ratio of 1:10 (w/w, with ~100% antigen adsorption, data not shown).

3.3.1 Stability of OVA/Alhydrogel® liquid vaccine

Initially, we evaluated the immunogenicity of the OVA/Alhydrogel® vaccine in liquid suspension (with 2% trehalose, w/v, to keep the composition identical to that in the dry vaccine powder). The liquid suspension was stored at 4° C, room temperature, 30° C, and 40° C for 3 months, and the particle size and size distribution of the vaccine were determined 1 and 3 months later. Shown in Figs. 1A-B are representative particle size distribution curves of the OVA/Alhydrogel® vaccine liquid suspension after 1 month (Fig. 1A) or 3 months (Fig. 1B) of storage in various temperatures. The representative particle

size distributions, expressed as X_{10}, X_{50}, and X_{90} of the OVA/Alhydrogel® vaccine liquid suspension after 1 month or 3 months of storage in various temperatures, are shown in Table 1. X_{10}, X_{50}, and X_{90} denote particle dimensions corresponding to 10%, 50%, and 90% of the cumulative undersize distribution. Aggregation was minimal after 1 month of storage, but became apparent after 3 months, as can be seen by the increase in X_{90} values (Fig. 1B, and Table 1). For example, the X_{90} values of the liquid vaccine suspension stored at 30° C increased from ~10 μm to ~42 μm (Table 1). For the liquid vaccine suspension stored at 30° C, there is a slight decrease in the X_{10} and X_{50} values from 1 month to 3 months of storage, but the X_{90} values are increased, indicating that smaller size particles aggregated to form bigger particles. This increase in X_{90} values skews the particle size distribution to the right. However, it is not clear why there was a slight decrease in the particle size for the OVA/Alhydrogel® liquid vaccine stored at 40° C (Table 1).

To evaluate the immunogenicity of the OVA/Alhydrogel® vaccine liquid suspension after 3 months of storage in room temperature or 4° C, mice were immunized (s.c.) with it, and the serum anti-OVA IgG levels induced were compared to that induced by freshly prepared OVA/Alhydrogel® vaccine liquid suspension. As shown in Fig. 1C, storing the OVA/Alhydrogel® vaccine liquid suspension in room temperature for 3 months led to significantly reduced anti-OVA IgG response, as compared to freshly prepared OVA/Alhydrogel® vaccine liquid suspension (Fig. 1C). We did not test the immunogenicity of the OVA/Alhydrogel® liquid vaccine stored at 30° C and 40° C because the vaccine does not contain any excipient that is known to improve the thermal stability of vaccines, which could explain the decrease in immunogenicity even when the liquid

vaccine was stored at 4° C (Fig. 1C). This is critical as there are aluminum salt-adjuvanted vaccines in liquid suspension that were reported to be stable at elevated temperatures (e.g. Havrix™ is stable at 37° C for 3 weeks; NeisVac-C™ is stable at 40° C for 1 month).(167-170) Therefore, the OVA/Alhydrogel® vaccine is a good candidate for us to test the immunogenicity of thin-film freeze-dried, aluminum salt-adjuvanted vaccine dry powder when exposed to temperatures outside the cold-chain. Data from a previous study reported that high concentrations of phosphate ion ($\geq$ 40 mM) increase the thermal stability of aluminum oxyhydroxide-adjuvanted hepatitis B vaccine (165). When preparing the OVA-adsorbed Alhydrogel® vaccine, we dissolved the OVA in a low concentration of PBS, and the final PBS concentration in the OVA/Alhydogel® liquid vaccine was 5 mM. As expected, the low concentration phosphate ion did not render the liquid vaccine thermostable (165).

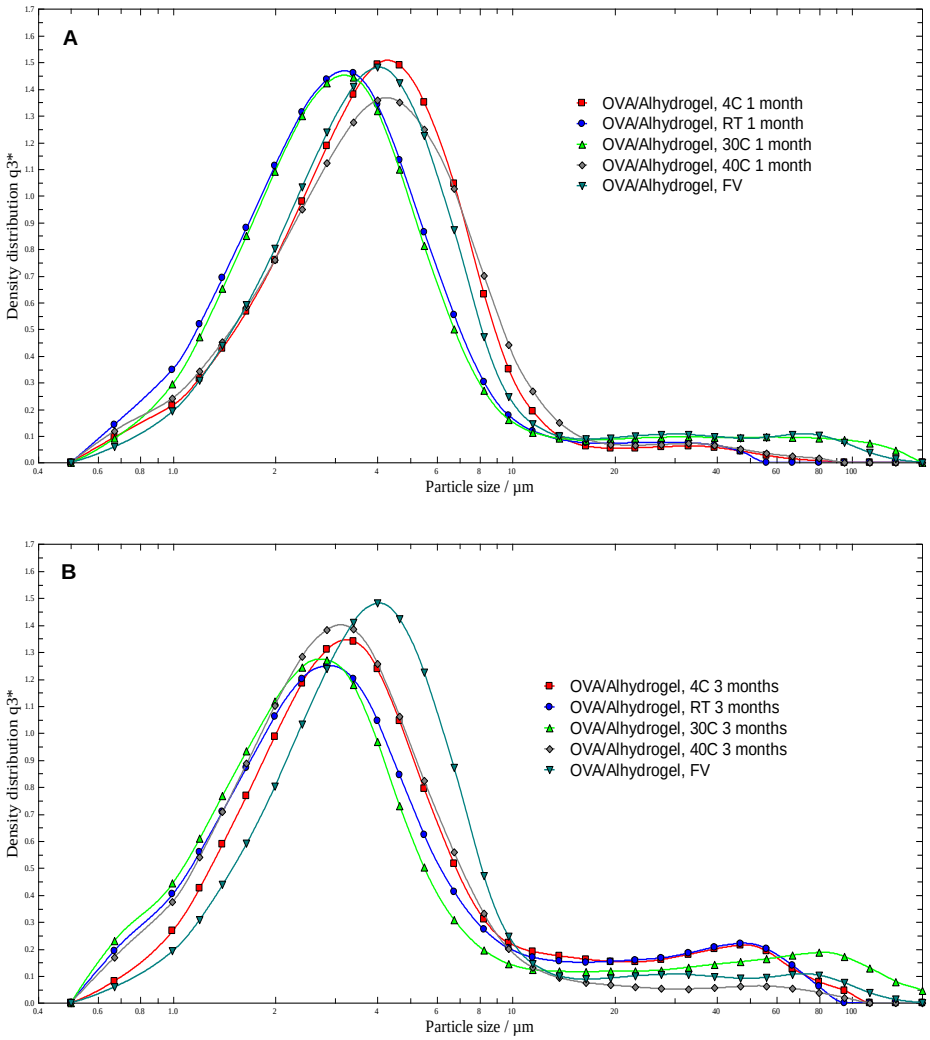


Figure 3.1 Effect of temperature on the stability of the OVA/Alhydrogel® liquid vaccine

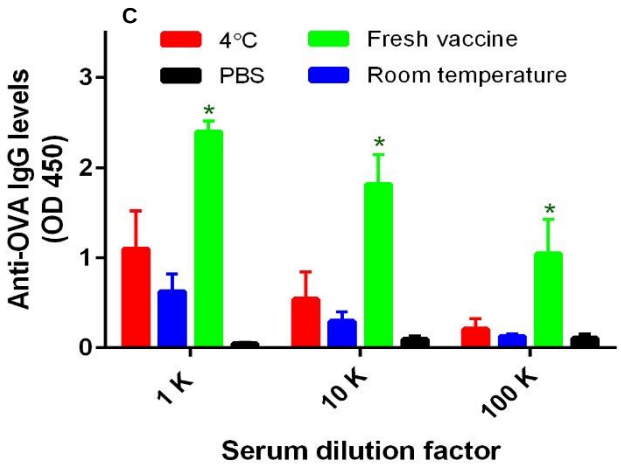


Figure 3.1 (Continued)

Representative particle size distribution curves of OVA/Alhydrogel® vaccine liquid suspension after 1 month **(A)** and 3 months **(B)** of storage in various temperatures. The experiment was carried out with three replicates with similar results (RT, room temperature; FV, fresh vaccine). **(C)** Serum anti-OVA IgG levels in mice immunized with OVA/Alhydrogel® liquid vaccine that was stored in 4° C or room temperature for 3 months. As controls, mice were injected with sterile PBS or freshly prepared OVA/Alhydrogel® vaccine (i.e. Fresh vaccine). Female BALB/c mice (n = 5) were injected (s.c.) on days 0, 14, and 28 with 5 μg of OVA per mouse. Total anti-OVA IgG levels in serum samples were measured about three weeks after the third dose. Data are mean ± S.D. (* $p < 0.05$, Fresh vaccine vs. others).

(A)

Liquid vaccine 1 month	X_{10} (µm)	X_{50} (µm)	X_{90} (µm)
OVA/Alhydrogel®, FV	1.56 ± 0.07	4.29 ± 0.68	10.77 ± 3.46
OVA/Alhydrogel®, RT	1.3 ± 0.04	3.03 ± 0.09	8.59 ± 1.57
OVA/Alhydrogel®, 4° C	1.65 ± 0.08	4.5 ± 0.66	10.35 ± 2.98
OVA/Alhydrogel®, 30° C	1.41 ± 0.04	3.23 ± 0.17	9.25 ± 1.56
OVA/Alhydrogel®, 40° C	1.5 ± 0.04	3.86 ± 0.55	9.25 ± 2.07

(B)

Liquid vaccine 3 month	X_{10} (µm)	X_{50} (µm)	X_{90} (µm)
OVA/Alhydrogel®, FV	1.56 ± 0.07	4.29 ± 0.68	10.77 ± 3.46
OVA/Alhydrogel®, RT	1.23 ± 0.05	3.19 ± 0.15	24.81 ± 15.07
OVA/Alhydrogel®, 4° C	1.35 ± 0.05	3.23± 0.16	17.38 ± 8.35
OVA/Alhydrogel®, 30° C	1.20 ± 0.06	3.07 ± 0.24	41.56 ± 23.29
OVA/Alhydrogel®, 40° C	1.04 ± 0.25	2.37 ± 0.51	6.38 ± 0.72

Table 3.1 Effect of storage temperatures on the particle size and size distribution of OVA/Alhydrogel® liquid vaccine suspension

Representative particle size distribution numbers expressed as X_{10}, X_{50}, and X_{90} of the OVA/Alhydrogel® vaccine liquid suspension after 1 month (table 1A) or 3 months (table 1B) of storage at various temperatures. Data are mean ± S.D. ($n \geq 3$). X_{10}, X_{50}, and X_{90} denotes to particle dimensions corresponding to 10, 50, and 90% of the cumulative undersize distribution.

3.3.2 Stability of OVA/Alhydrogel® vaccine dry powder

We then stored the thin-film freeze-dried OVA/Alhydrogel® vaccine powder in room temperature, 30° C, and 40° C. The dry powder was reconstituted 1, 3, and 6 months later to evaluate the particle size and size distributions, and/or the immunogenicity of the vaccine. Shown in Fig. 2 are representative particle size distribution curves of the OVA/Alhydrogel® vaccine reconstituted from thin-film freeze-dried powder that was stored in room temperature, 30° C, or 40° C for 1 month (Fig. 2A), 3 months (Fig. 2B), or 6 months (Fig. 2C), respectively. Particle aggregation was not detected after 1 month of storage in all three temperatures (Fig. 2A, Table 2). Slight particle aggregation was detected after 3 months of storage at 40° C, as can be seen by the increase of X_{90} values from 20 μm to 51 μm (Fig. 2B, Table 2). After 6 months of storage, particle aggregation became noticeable in all three temperatures, especially 40° C (Fig. 2C, Table 2). Similar to the particle size distribution in the liquid vaccine after storage, the increase in the particle size in the dry powder after storage can be seen in the X_{90} values only. This is likely because as the particles aggregate, there is a slight increase in the X_{90} value. An increase in the X_{50} value would indicate the aggregation is more severe, as will be showed in the case of subjecting the vaccine to repeated freezing-and-thawing cycles in a later section.

The chemical stability of the OVA antigen in the OVA/Alhydrogel® vaccine dry powder was also monitored. SDS-PAGE data show that after the OVA/Alhydrogel® vaccine dry powder was stored for 1 month in different storage temperatures, the OVA desorbed from the OVA/Alhydrogel® dry powder was similar to the OVA desorbed from freshly prepared OVA/Alhydrogel® liquid suspension (data not shown). However, after 6

months of storage, especially at 40° C, the band intensity of the OVA desorbed from the OVA/Alhydrogel® dry powder became much weaker than that desorbed from freshly prepared OVA/Alhydrogel® liquid suspension (data not shown), which may be attributed to protein chemical degradation and/or decreased desorption of OVA from Alhydrogel® due to tight binding. There are reports that storing vaccines for an extended period of time may increase the tightness of the binding of the antigens to Alhydrogel® (171-173).

Shown in Fig. 3 are the anti-OVA IgG levels in mice that were immunized with OVA/Alhydrogel® vaccine reconstituted from dry powder that was stored in various temperatures for 1 month (Fig. 3A) or 3 months (Fig. 3B). Clearly, after 3 months of storage, the serum anti-OVA IgG levels in mice immunized with OVA/Alhydrogel® reconstituted from the thin-film freeze-dried powder were not significantly different from that induced by freshly prepared OVA/Alhydrogel® vaccine (Fig. 3). Also, the serum anti-OVA IgG levels for the dry vaccine powders stored for 1 month and 3 months are not different. Due to the physical and chemical instability (e.g. particle aggregation, potential antigen degradation) detected after the OVA/Alhydrogel® dry powder was stored in various temperatures, especially at 40° C, it became unnecessary to test the immunogenicity of the vaccine dry powder after 6 months of storage.

It was previously reported that converting vaccines from liquid to dry powder can render the otherwise fragile vaccine stable in ambient temperatures (129, 174-179). The OVA/Alhydrogel® particles in the thin-film freeze-dried powder are embedded in a glass of trehalose with a glass transition temperature (i.e. T*g*) of 120° C. The T*g* is significantly higher than highest temperature (i.e. 40° C) in which the vaccine dry powder was stored,

allowing the vaccine to remain in a low-mobility, glassy state. However, it is worth pointing out that in the present study, while the temperatures in which the OVA/Alhydrogel® vaccine was stored was controlled, the moisture content in the powder increased with the extended period of storage, and was higher in higher temperature. For example, after 3.5 months of storage, the moisture content in the powders stored at 40° C, 30° C, and room temperature increased from the initial value of 1-3% to 7.7 ± 1.9%, 6.0 ± 0.9%, 5.4 ± 0.5%, respectively ($n = 6\text{-}9$, $p < 0.05$, values at 40° C vs. in room temperature). It is thus expected that the vaccine dry powder may potentially be stored in room temperature or above for a longer period of time, if the moisture content of the dry powder is better controlled during storage (129). Alternatively, an excipient that is less hygroscopic than trehalose and has a higher T*g* value, e.g. dextran, could be potentially used to improve the storage stability (176). For instance, recently Kunda et al. showed that modified packaging system (inert N_2 gas, oxygen scavengers, and desiccant sachet) can be used to control the moisture content to increase the stability of the dry powder vaccines in elevated temperatures (180). Nonetheless, data in Figure 3 show that thin-film freeze-drying offers a potentially viable approach to manage aluminum salt-adjuvanted vaccines in CTC, because exposing the thin-film freeze-dried vaccine powder to temperatures as high as 40° C for up to 3 months did not cause significant decrease in the immunogenicity of the vaccine upon reconstitution.

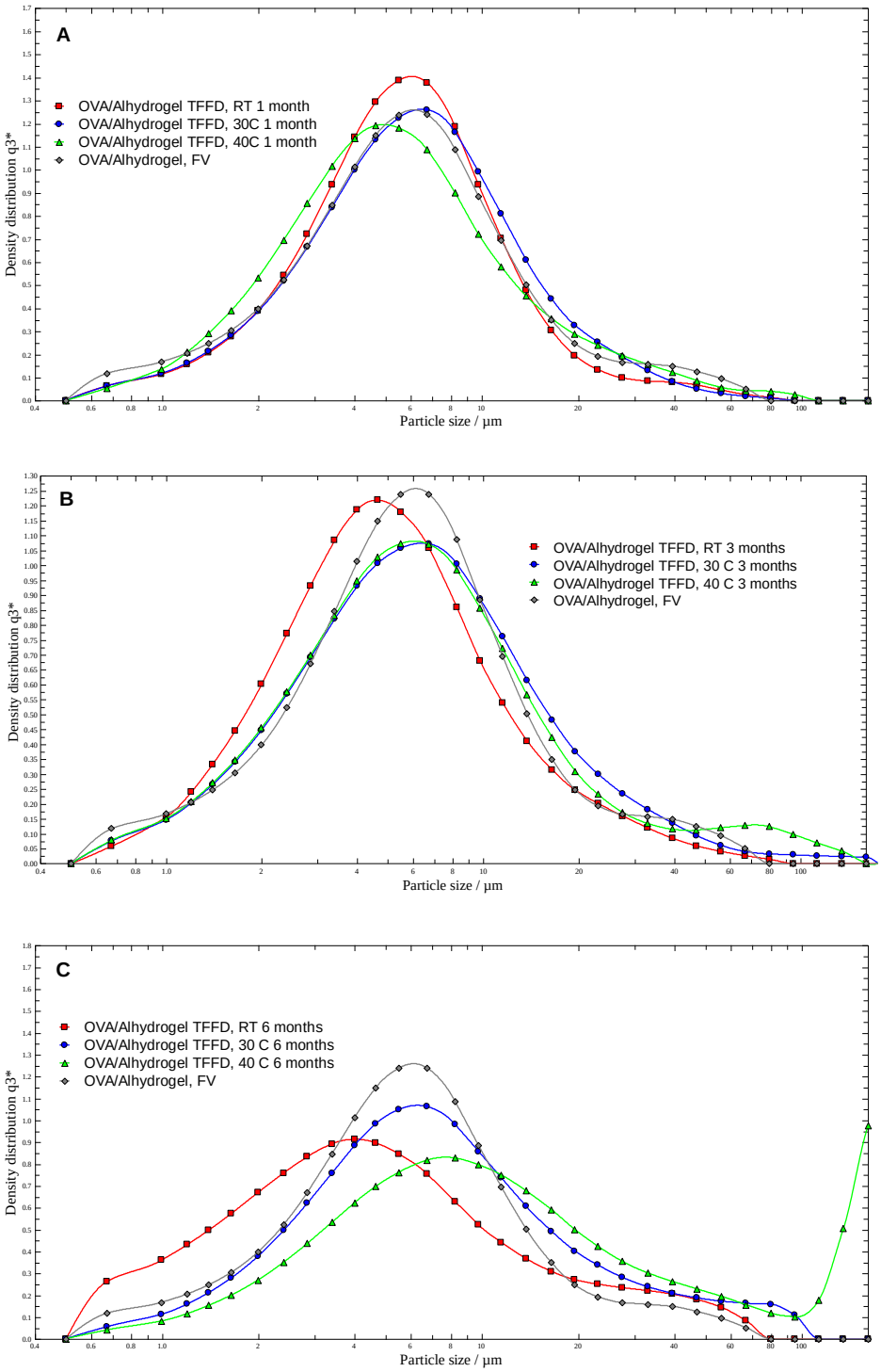


Figure 3.2 Effect of temperature on particle size distribution of the OVA/Alhydrogel® vaccine dry powder

Figure 3.2 (Continued)

Representative particle size distribution curves of OVA/Alhydrogel® vaccine dry powder after stored in various temperatures for 1 month (A), 3 months (B), and 6 months (C) and then reconstituted (TFFD, thin-film freeze-dried vaccine powder; RT, room temperature; FV, fresh vaccine). The experiment was carried out with three replicates with similar results.

(A)

TFFD 1 month	X_{10} (µm)	X_{50} (µm)	X_{90} (µm)
OVA/Alhydrogel®, FV	2.36 ± 0.005	6.37 ± 0.02	13.79 ± 0.11
OVA/Alhydrogel®, RT	2.14 ± 0.02	5.96 ± 0.46	15.39 ± 2.54
OVA/Alhydrogel®, 30° C	1.97 ± 0.21	5.44 ± 0.87	13.89 ± 3.42
OVA/Alhydrogel®, 40° C	2.13 ± 0.33	6.25 ± 1.49	20.37 ± 4.41

(B)

TFFD 3 month	X_{10} (µm)	X_{50} (µm)	X_{90} (µm)
OVA/Alhydrogel®, FV	2.36 ± 0.005	6.37 ± 0.02	13.79 ± 0.11
OVA/Alhydrogel®, RT	1.73 ± 0.05	4.76 ± 0.06	15.28 ± 0.55
OVA/Alhydrogel®, 30° C	1.95 ± 0.04	6.29 ± 0.31	28.24 ± 15.39
OVA/Alhydrogel®, 40° C	1.88 ± 0.01	6.17 ± 0.20	51.17 ± 28.14

(C)

TFFD 6 month	X_{10} (µm)	X_{50} (µm)	X_{90} (µm)
OVA/Alhydrogel®, FV	2.36 ± 0.005	6.37 ± 0.02	13.79 ± 0.11
OVA/Alhydrogel®, RT	1.10 ± 0.01	3.78 ± 0.36	16.44 ± 5.01
OVA/Alhydrogel®, 30° C	2.19 ± 0.03	6.86 ± 0.18	30.04 ± 5.49
OVA/Alhydrogel®, 40° C	2.20 ± 0.32	7.53 ± 1.75	47.03 ± 44.14

Table 3.2 Effect of storage temperatures on the particle size and size distribution of OVA/Alhydrogel® dry vaccine powder

Representative particle size distribution numbers expressed as X_{10}, X_{50}, and X_{90} of the OVA/Alhydrogel® dry vaccine powder after 1 month (table 2A), 3 months (table 2B), or 6 months (table 2C) of storage at various temperatures. Data are mean ± S.D. ($n \geq 3$). X_{10}, X_{50}, and X_{90} denotes to particle dimensions corresponding to 10, 50, and 90% of the cumulative undersize distribution.

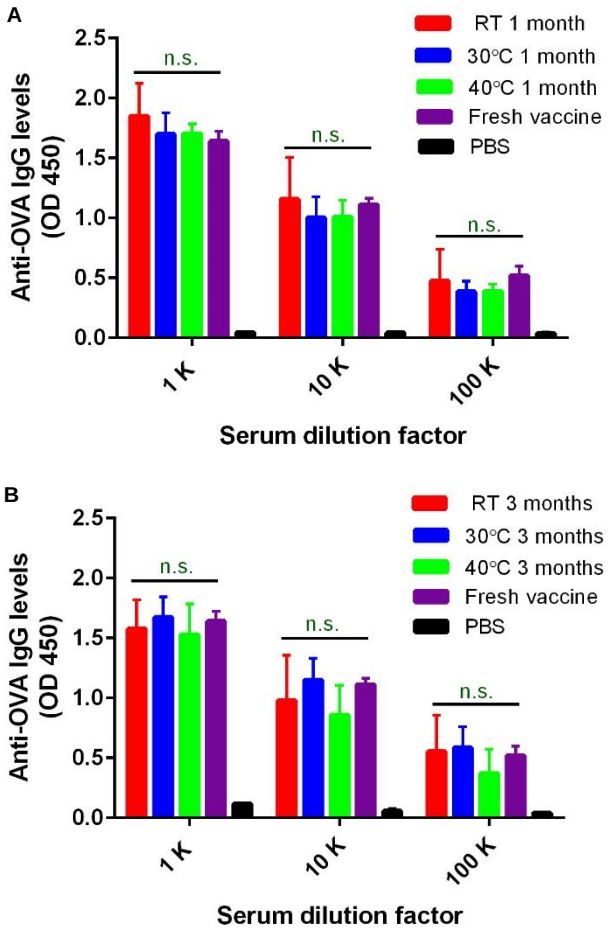


Figure 3.3 Effect of temperature on the immunogenicity of the dry vaccine powder

Serum anti-OVA IgG levels in mice immunized with OVA/Alhydrogel® vaccine dry powder that was stored at 40° C, 30° C, or room temperature for 1 month (A) or 3 months (B) and then reconstituted. As controls, mice were injected with sterile PBS or freshly prepared OVA/Alhydrogel® vaccine (i.e. Fresh vaccine). Female BALB/c mice (n = 5) were injected (s.c.) on days 0, 14, and 28 with 5 μg of OVA per mouse. Total anti-OVA IgG levels in serum samples were measured about three weeks after the third dose. Data are mean ± S.D. (n.s., not significant)

3.3.3 Stability of OVA/Alhydrogel® dry powder vaccine to freezing

The risk of exposing a vaccine to freezing temperatures during transport and/or storage exists regardless whether the vaccine is managed in cold-chain, CTC, or in ambient temperatures. Converting aluminum salt-adjuvanted vaccines from a liquid suspension to dry powder by thin-film freeze-drying is expected to render the vaccine insensitive to freezing. The OVA/Alhydrogel® particles in the thin-film freeze-dried powder are embedded in a glass of trehalose with a T*g* value of 120° C (159). Trehalose limits the mobility of the aluminum salt particles by forming glass during freezing (122), and we have previously shown using transmission electron microscopy that the OVA-adsorbed aluminum hydroxide particles are embedded in the bulk structure of trehalose, which likely prevents the particles from interacting with one another during freeze-drying process.(159) Similarly, the trehalose glass is expected to make it difficult, if possible, for any subsequent slow freezing process to break the lattice that binds the antigen (i.e. OVA) to the Alhydrogel® and/or for the separated Alhydrogel® particles, if any, to aggregate to form larger and heavier particles. This explains our previous finding that subjecting the thin-film freeze-dried powder of aluminum salt-adjuvanted vaccines, e.g. GSK's Engerix-B™, to repeated slow freezing-and-thawing cycles does not cause particle aggregation upon reconstitution (159).

In the present study, we further tested whether the immunogenicity of thin-film freeze-dried OVA/Alhydrogel® vaccine is affected after it was subjected to three cycles of repeated slow freezing-and-thawing and reconstitution. Changes (or lack of them) in the immunogenicity of a vaccine are difficult to predict. Any slight change in the physical and

chemical characteristics of the antigen (i.e. OVA) and/or the vaccine if inadvertently subjected to freezing can potentially lead to a decrease in the immunogenicity of the vaccine. Shown in Table 3 are the particle size distributions of the OVA/Alhydrogel® vaccine in liquid suspension or as dry powder, before and after being subjected to three cycles of slow freezing-and-thawing (i.e. F/T stress). As expected (159), after the OVA/Alhydrogel® vaccine in liquid suspension was subjected to three cycles of slow freezing-and-thawing, the X_{50} value increased by 8.6-fold (i.e. from 7.5 to ~64 μm) (Table 3), but subjecting the thin-film freeze-dried OVA/Alhydrogel® powder to the same three cycles of freezing-and-thawing did not cause any significant change in the particle size and size distribution (Table 3). Data in Fig. 4 show that the serum anti-OVA IgG levels in mice immunized with OVA/Alhydrogel® dry powder that was subjected to the slow freezing-and-thawing cycles are not significantly different from that in mice that were immunized with OVA/Alhydrogel® dry powder that was not subjected to the freeze-and-thaw cycles, or in mice that were immunized with freshly prepared OVA/Alhydrogel® liquid vaccine (Fig. 4). On the other hand, the serum anti-OVA IgG levels in mice that were immunized with OVA/Alhydrogel® liquid vaccine that was subjected to three cycles of slow freezing-and-thawing are significantly lower than that in mice immunized with the freshly prepared OVA/Alhydrogel® liquid vaccine (Fig. 4). It is clear that the OVA/Alhydrogel® liquid vaccine is sensitive to freezing, because freezing caused significant particle aggregation (Table 3) and significantly reduced its immunogenicity (Fig. 4). It was reported previously that subjecting OVA protein to 5 repeated freezing-and-thawing cycles induces OVA denaturation in a concentration dependent manner (160). However, it is unclear to what

extent the reduction in the immunogenicity (i.e. anti-OVA IgG levels) of the OVA/Alhydrogel® liquid vaccine after it was subjected to repeated freezing-and-thawing cycles can be attributed to OVA denaturation in the present study. It is certain though that converting the OVA/ Alhydrogel® vaccine from liquid suspension to dry powder by thin-film freeze-drying can protect the vaccine from damages induced by slow freezing (and thawing) (Fig. 4).

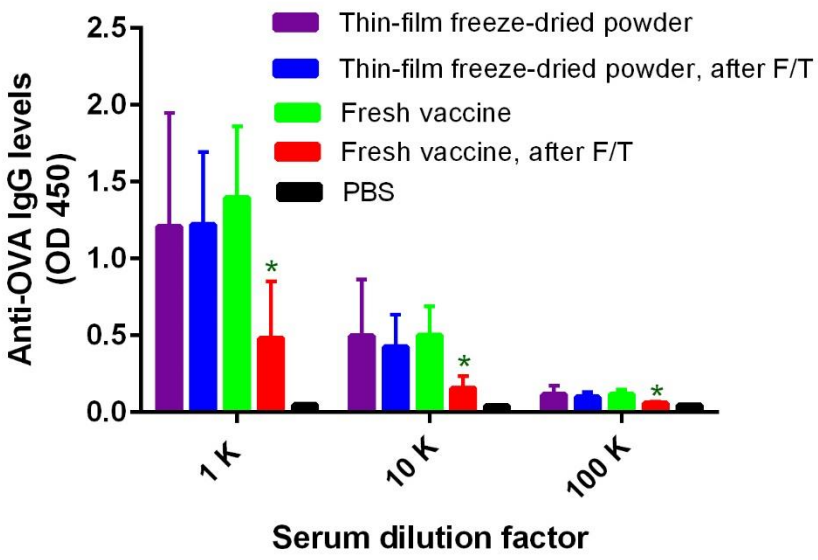


Figure 3.4 Effect of repeated freezing-and-thawing on the immunogenicity of the dry vaccine powder

Serum anti-OVA IgG levels in mice immunized with dry powder that was subjected to three cycles of freezing-and-thawing (F/T) and then reconstitution or the same OVA/Alhydrogel® liquid vaccine subjected to the same three cycles of freezing-and-thawing. As controls, mice were immunized with freshly prepared OVA/Alhydrogel® liquid vaccine or dry powder upon reconstitution. Female BALB/c mice (n = 5) were injected (s.c.) on days 0, 14, and 28 with 5 μg of OVA per mouse. Total anti-OVA IgG levels in serum samples were measured about three weeks after the third immunization. Data are mean ± S.D. (* $p < 0.05$, Fresh vaccine, after F/T vs. other immunized groups).

	X_{10} (µm)	X_{50} (µm)	X_{90} (µm)
OVA/Alhydrogel® FV	2.13 ± 0.35	7.51 ± 1.92	26.66 ± 11.35
OVA/Alhydrogel® TFFD	2.83 ± 0.06	7.58 ± 0.21	22.27 ± 2.15
OVA/Alhydrogel® FV, F/T	12.35 ± 0.48	64.31 ± 1.86	137.39 ± 3.31
OVA/Alhydrogel® TFFD, F/T	1.36 ± 0.05	5.01 ± 0.29	16.15 ± 2.19

Table 3.3 Effect of repeated freezing-and-thawing on the particle size and size distribution of OVA/Alhydrogel® vaccine dry powder

The particle size distributions of freshly prepared OVA/Alhydrogel® liquid vaccine (i.e., OVA/Alhydrogel® FV) or thin-film freeze-dried OVA/Alhydrogel® vaccine powder after reconstitution (i.e., OVA/Alhydrogel® TFFD) were measured before or after they were subjected to 3 cycles of freeze-and-thaw (F/T). X_{10}, X_{50}, and X_{90} denote particle dimensions corresponding to 10%, 50%, and 90% of the cumulative undersize distribution. Data are mean ± SD ($n \geq 3$).

The development of aluminum salt-adjuvanted vaccines that can be managed in CTC or potentially in ambient temperatures and are not sensitive to freezing will not only help decrease the loss of global vaccine supplies due to breaches in cold-chain, but also avoid the unintentional administration of damaged, suboptimal vaccines to patients. Data in the present study confirm that TFFD is a technology that can potentially enable the development of such vaccines. The traditional TFFD process involves dropping droplets of liquid (e.g. a liquid suspension of vaccine) over a cryogenically-cooled substrate, e.g. the surface of a rotating cooled metal drum, to form frozen thin-film upon impact (70). We also showed that the inner surface of silanized glass vials can be used as a cryogenically-cooled substrate as well to drop liquid vaccine droplets onto to form frozen thin-films upon impact. Subsequently, the glass vials with the frozen vaccine thin films can be subjected to standard lyophilization to remove water. As shown in Fig. 5, the particle size distribution of OVA/Alhydrogel® reconstituted from the dry powder prepared by single vial TFFD is similar to that of the freshly prepared OVA/Alhydrogel® liquid vaccine or that reconstituted from the dry powder prepared by the traditional TFFD. As expected (60, 143-147), slow tray-freezing followed by lyophilization led to significant aggregation of the OVA/Alhydrogel® vaccine (Fig. 5). The feasibility of TFFD in single vials is expected to allow the TFFD technology to be readily adopted in existing freeze-drying facilities with minimal modifications.

It is worth mentioning that besides TFFD, other freezing and/or drying techniques are also explored to convert protein products into dry powders (61-64). Spray freeze-drying has been particularly studied to convert aluminum salt-adjuvanted vaccines into dry

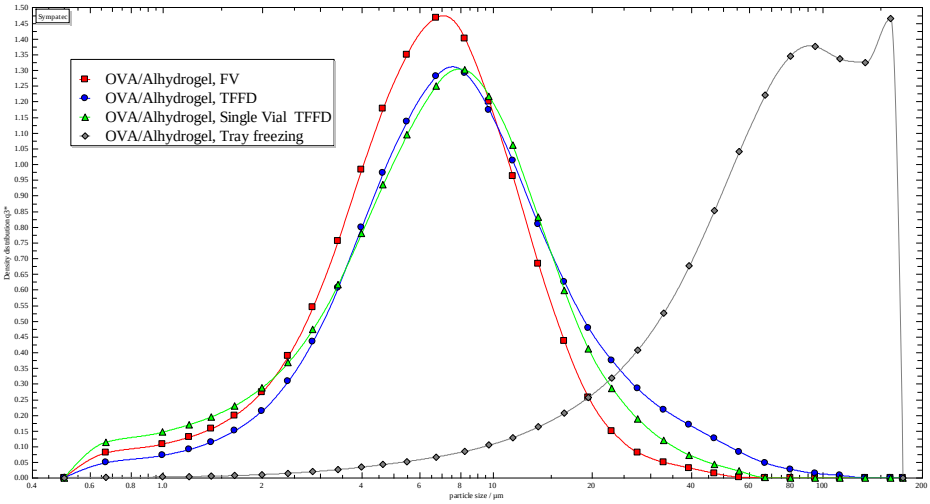


Figure 3.5 Single vial thin-film freeze-drying

Representative particle size distribution curves of OVA/Alhydrogel® vaccine before (i.e. OVA/Alhydrogel®, FV, □) and after it was subjected to thin-film freezing on a pre-cooled rotating metal drum (OVA/Alhydrogel®, TFFD, ○), thin-film freeze-drying on the inner surface of a silalized glass vial (OVA/Alhydrogel®, single vial TFFD, Δ), or conventional tray-freezing (OVA/Alhydrogel®, Tray freezing, ◊), and then lyophilization and reconstitution (FV, fresh vaccine; TFFD, thin-film freeze-drying). The experiment was carried out with three replicates with similar results.

powder using various excipients (e.g. mixture of glycine, mannitol, and dextran up to 10% w/v) (67, 181). Besides the approach of converting aluminum salt-adjuvanted vaccine from liquid suspension to dry powder, there are also reports that certain excipients such as high concentrations of phosphate anions and histidine can help improve the thermal stability of vaccine. In addition, polyols such as propylene glycol and glycerol can help make vaccine insensitive to freezing (157, 164-166). Neither one of those approaches alone will likely be universally applicable to all vaccines, but it is certain, as demonstrated in the present study, that technologies are available to make aluminum salt-adjuvanted vaccines thermostable.

3.4 CONCLUSION

In conclusion, using a model vaccine prepared by adsorbing OVA onto Alhydrogel®, we confirmed that the vaccine dry powder prepared using TFFD is stable in temperatures as high as 40°C for 3 months and no longer sensitive to slow freezing. Global immunization program may potentially benefit by integrating TFFD into vaccine preparations.

Chapter Four

Novel dry powder formulation of aluminum salt-adjuvanted vaccine for intranasal vaccination

4.1 INTRODUCTION

Adjuvants are needed for newer generation vaccines, such as protein subunit vaccines to elicit a strong immune response (182). Some insoluble aluminum salts, e.g. aluminum (oxy)hydroxide and aluminum (hydroxy)phosphate, are used in many currently licensed vaccines as adjuvants and possess excellent safety profiles (140, 141). A major limitation with aluminum salt-adjuvanted vaccines is that they must be maintained in cold-chain (2-8 °C) during transport and storage (148). Accidentally subjecting the vaccines to freezing compromises their potency and/or efficacy. To overcome this issue, previously we reported that thin-film freeze-drying (TFFD) can be used to convert aluminum salt-adjuvanted vaccines from liquid to dry powder without causing particle aggregation or decreasing the immunogenicity following reconstitution (159, 183). Importantly, the resultant dry powder vaccine can be stored in temperatures as high as 40 ^{0}C for an extended period of time (183).

Aluminum salt-adjuvanted vaccines are generally administered by subcutaneous, intradermal, or intramuscular injection. However, reconstituting the dry powder vaccine before injection has various limitations such as the need for sterile water for injection, the need for trained medical personnel, and the increased chance of error made when reconstituting the powder and filling syringes. The nasal route for immunization offers

some interesting opportunities. Almost all infectious agents enter the body through the mucosal surfaces (80), and the nasal mucosa is often the first point of contact for inhaled pathogens. Therefore, ideally, to more effectively protect against inhaled pathogens, vaccines should be administered via the nasal mucosal surface to induce mucosal immunity to prevent infectious agents from entering the host (81). Besides enabling vaccines to induce both mucosal and systemic immune responses (82, 83), intranasal immunization has several other advantages as well. For example, the nose tissue is easily accessible and highly vascularized, and can be used in the case of epidemics for mass vaccination. In addition, nasal immunization enables needle-free, non-invasive delivery of vaccines with the possibility of self-immunization.

Previously, it was thought that aluminum salt-based adjuvants are not capable of potentiating mucosal immune responses when given intranasally, and results from prior studies were ambiguous (94-97). However, data from our recent studies clearly showed that intranasal immunization with antigens adsorbed on Alhydrogel® induces significantly stronger antigen-specific immune responses, both systemically and in nasal and lung mucosa, as compared to intranasal immunization with the antigens alone (98). In the present study, we tested the feasibility of administering aluminum salt-adjuvanted dry powder vaccine prepared using thin-film freeze-drying method directly via the nasal route to induce specific mucosal and systemic immune responses in a rat model. Others have administered dry powder vaccines intranasally in animal models (184, 185), and even in clinical trials (186, 187), but these vaccines do not contain aluminum salts as adjuvants. Aluminum salt-adjuvanted dry powder vaccine preparations have also been administered

in animal model in a needle-free method, bur by needle-free intradermal injection (63, 188). In our opinion, this would be the first report of successfully using aluminum salt-adjuvanted vaccine dry powder for intranasal immunization.

4.2 MATERIALS AND METHODS

4.2.1 Preparation of dry powder vaccine

The ovalbumin (OVA)-Alhydrogel® dry powder vaccine was prepared as described previously (159, 183). Briefly, OVA-Alhydrogel® liquid vaccine was prepared by adding 10 mL of Alhydrogel® (10 mg/mL aluminum, manufactured by Brenntag, and supplied by InvivoGen, San Diego, CA) into a 50-mL tube followed by the addition of 10 mL of an OVA solution (1 mg/mL in 0.9% w/v saline solution), and 200 mg of trehalose (Sigma-Aldrich, St. Louis, MO) to obtain a final formulation with 2% (w/v) of trehalose, ~1% (w/v) of Alhydrogel®, and 0.5 mg/mL of OVA. The vaccine suspension was converted into a dry powder using previously reported thin-film freeze-drying method (159, 183). The powder was dried using a VirTis AdVantage Bench Top Lyophilizer (The VirTis Company, Inc. Gardiner, NY). Lyophilization was performed over 72 h at pressures less than 200 mTorr, while the shelf temperature was gradually ramped up from −40 °C to 26 °C. After lyophilization, the solid dry powder vaccine was transferred into a sealed container and stored in a desiccator.

4.2.2 Physicochemical characterization

4.2.2.1 Particle size analysis

Geometric diameter of the dry powder vaccine was determined by low angle light scattering using a Malvern Spraytec® (Malvern, UK) outfitted with an inhalation cell and without an induction port. The nasal dry powder delivery device filled with the powder

was secured to the mouth of the induction port by a molded silicone adapter. The measurement was done at a flow rate of 25 L/min, providing a 2 kPa pressure drop across the device. Data acquisition took place over 4 s and only when laser transmission dropped below 95%.

4.2.2.2 Powder morphology and uniformity of distribution

Scanning electron microscopy (SEM) attached to energy-dispersive spectroscopy (EDS) was applied to understand the structure and morphology of the dry powder vaccine, and to determine the uniformity of distribution of the dry powder vaccine. Hitachi S-5500 SEM (Hitachi High Technologies America, Inc., Pleasanton, CA) equipped with EDS was used at 10 kV accelerating voltage after sputter-coating the specimen with silver for 30 s in vacuum. Images at different magnifications are photographed from SEM, and EDS plots showing elemental mapping are reported.

4.2.2.3 Flow properties

The tapped density of the dry powder vaccine was measured according to a method adapted from the United States Pharmacopeia (USP) <616> method I. An adaptation was made due to the limited supply of powder for testing, where a 100-mL graduated cylinder was replaced by a 5-mL graduated cylinder. Hausner ratio and Carr's compressibility index were calculated for each formulation based on USP guidelines. Measurements of the static angle of repose of the dry powder vaccine were conducted as per USP <1174>. Approximately 10 mL of powder was measured using a funnel and a flat collection surface.

4.2.3 Aerodynamic assessment of the dry powder vaccine

Aerodynamic assessment of the dry powder vaccine intended for nasal administration was performed in a nasal cast model. The casts were made from CT scans of human adult noses (189) and can be divided into four sections representing the anterior region making up the vestibule (V) and nasal valve area; lower, middle and upper turbinates collectively called posterior nasal cavity (P); nasopharynx region (N); and the post-nasal fraction (F), which can either be collected in a cup if the model is operated without simulated inspiration airflow, or a removable filter is connected to the nasopharynx section if operated at a normal nasal breathing of 15 L/min. The cast was coated with 1% Tween 20 in methanol and allowed to dry prior to deposition studies to mimic the mucus layer and minimize particle bounce (190). The formulations were administered to the cast with a nasal dry powder delivery device prepared in our laboratory. The spray administration angle was set at 60° from horizontal, and the tip of the device was inserted at a distance of 5 mm. After administration, the cast was disassembled and all parts were washed with 2% nitric acid carefully to collect the deposited fractions. Aluminum content was determined using a Varian 710-ES Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES) in the Civil Architectural and Environmental Engineering Department at The University of Texas at Austin. The deposition profile is shown as % deposition of recovery in different sections of the cast model.

4.2.4 Animal studies

All animal studies were conducted following the U.S. National Research Council Guidelines for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee at The University of Texas at Austin approved the animal protocol. Female Sprague-Dowley rats, 6-8 weeks of age, were from Charles River Laboratories Inc. (Wilmington, MA). Rats were dosed intranasally on days 0, 14 and 28 with the OVA-Alhydrogel® dry powder vaccine (IN Powder, n = 4) using our nasal dry powder delivery device. The rats were anesthetized and placed on their back at an angle of 45°. The exit diffuser of the device loaded with the vaccine powder was placed in the right nostril of the anesthetized rat. The plunger of the syringe that contains 1 mL of air was depressed to create a powder plume of the vaccine in the nasal cavity. As controls, rats were also intranasally dosed with the OVA-Alhydrogel® liquid vaccine (IN Liquid, n = 4), saline (n = 5), or subcutaneously (s.c.) injected with the OVA-Alhydrogel® liquid vaccine (SC Liquid, n = 4). For intranasal dosing of the liquid vaccine and normal saline, rats were placed on their back at an angle of 45° after anesthesia. The liquid vaccine or normal saline was administered using a fine pipette tip into the external nares with 10 µL volume in each nostril. The dose of OVA was 20 µg per rat, and 400 µg for Alhydrogel®. Four weeks after the third dose, rats were euthanized to collect blood, nasal wash, and bronchoalveolar lavage (BAL). Nasal wash and BAL were collected as previously described using 500 µL of sterile PBS (191).

The anti-OVA IgG and IgA levels in serum samples, nasal washes, and BAL samples were determined using enzyme-linked immunosorbent assay (ELISA) (116).

Briefly, EIA/RIA flat bottom, NUNC Maxisorp, 96-well plates (Thermo Fisher) were coated with 1 ng/μL of OVA solution in carbonate buffer (0.1 M, pH 9.0) overnight at 4 °C. Plates were blocked with horse serum for one hour before adding the blood serum. Horse radish peroxidase (HRP)-labeled goat anti-mouse immunoglobulin (IgG or IgA, 5000-fold dilution, Southern Biotechnology Inc., Birmingham, AL) was added into the plates, and the presence of bound antibody was detected in the presence of 3,3′,5,5′-tetramethylbenzidine solution (TMB, Sigma–Aldrich). The absorbance was read at 450 nm.

Brain tissues were collected from the rats upon euthanasia. The tissues were weighed, desiccated at 60 °C for 12 h, and then incinerated with nitric acid (6.6 N) at 60 °C for 15 h. Aluminum content was determined using an Agilent 7500ce quadruple Inductively Coupled Plasma attached with Mass Spectrometer (ICP-MS) in the Department of Geological Sciences at The University of Texas at Austin. ICP-MS has a detection limit of 1 ng/L (parts per trillion) for aluminum (192). The aluminum levels are normalized to the dry weight of the brain tissues.

Noses of rats immunized by the intranasal (dry powder and liquid vaccine) and subcutaneous routes were prepared for histological examination. For this, the noses were separated, immobilized and decalcified. Afterwards the samples were sliced horizontally from the nostrils to the nasopharynx and slices were stained with hematoxylin and eosin (H&E) stain for histological evaluation. Microscopical pictures of the nasal cross section and close ups of the epithelium have been taken.

4.2.5 Statistics

Statistical analyses were completed by performing two-tailed Student's t-test for two-group analysis or one-way ANOVA followed by Tukey's post hoc analysis for multiple group comparisons (GraphPad Prism 7 software, La Jolla, CA). A p value of $\leq$ 0.05 (two-tail) was considered significant.

4.3 RESULTS AND DISCUSSION

Insoluble aluminum salt-adjuvanted vaccines contribute to more than 50% of global vaccines. Although search for alternative vaccine adjuvants continues today, insoluble aluminum salt-based adjuvants remain the preferred choice of adjuvants in vaccine formulations. Aluminum salt-adjuvanted vaccines, however, are particularly sensitive to unintentional freezing and/or heat during transport and storage, and have to be maintained in cold-chain (2-8 °C). Unfortunately, breaching of the cold-chain is a common place, and not resource limited (183). To overcome this drawback, we previously reported a method to convert aluminum salt-adjuvanted vaccines from liquid to dry powder without causing particle aggregation or decreasing the immunogenicity following reconstitution. However, immunization using hypodermic needles attached to syringe filled with liquid vaccine reconstituted from the dry powder has some inherit limitations. Data from our recent study showed that it is feasible to administer antigens adsorbed on an aluminum salt (i.e. Alhydrogel®) intranasally to induce specific systemic and mucosal immune responses (98). This finding led us to hypothesize that administering an aluminum-salt adjuvanted dry powder vaccine directly to the nose cavity will induce specific systemic and mucosal immune responses. We tested the hypothesis in a rat model with OVA as a model antigen adsorbed on Alhydrogel®, the international standard preparation of aluminum (oxy)hydroxide gel. OVA-Alhydrogel® liquid vaccine was converted to a dry powder using our previously reported thin-film freeze-drying method using 2% of trehalose as a cryoprotectant. The dry powder does not contain any known mucoadhesive agent.

4.3.1 Flow properties of the OVA-Alhydrogel® dry powder vaccine

The flow properties measure the cohesive forces of a powder. In this study, the dry powder vaccine was prepared to explore the feasibility of intranasal administration, not their flow properties per se. However, the flow properties do affect the performance of the final product (193). Also, because nasal delivery requires a fluidization of the powder bed, it is conceivable that the flow properties of the dry powder vaccine could affect the emitted dose from the nasal delivery device and the deposition of the vaccine in the nasal cavity.

The bulk and tapped densities of the dry powder was 0.040 ± 0.003 and 0.051 ± 0.007 g/mL, respectively (Table 1). The Hausner ratio and Carr's compressibility index of the dry powder vaccine was calculated to be 1.28 ± 0.07 and 21.80 ± 4.25%, respectively, indicating that the flow property of the powder is 'passable'. The Angle of repose of the dry powder was 25.94 ± 6.30°, which indicates a good flow property. The discrepancy in the flow property between the two methods of measurement could be explained by the porous and brittle nature of the thin-film freeze-dried vaccine powder, making it more compressible.

Bulk Density (g/mL)	Tap Density (g/mL)	Carr's Compress. Index (CCI)	Hausner Ratio	Static Angle of Repose (°)
0.040 ± 0.003	0.051 ± 0.007	21.80 ± 4.25	1.28 ± 0.07	25.94 ± 6.30

Table 4.1 Physical characterization of the TFFD dry powder vaccine

Tapped density of TFFD dry powder was measured according to a method adapted from USP <616> method I using a Varian Tapped Density Tester (Varian, Palo Alto, CA). An adaptation was made due to the limited supply of powder for testing where 100 mL graduated cylinder was replaced by a 5-mL graduated cylinder. Hausner ratio and Carr's (Compressibility) index were calculated for each formulation based on USP guidelines.

4.3.2 Powder morphology and uniformity of distribution

According to USP chapter <905>, each unit in a batch should have a drug substance content within narrow range of the claimed limit, which is referred to as uniformity of the dosage unit. The International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) Q6B also provides specifications for biotechnological and biological products to perform uniformity test of the dosage units of the biologicals. SEM/EDS has an X-ray spectrometer attached to SEM, which allows elemental analysis in addition to SEM. SEM/EDS could be employed to determine the qualitative distribution of the OVA-Alhydrogel® dry powder vaccine by taking advantage of the elemental aluminum in the formulation. This analysis gives an indication of how the vaccine is distributed in the dry powder form. Three random areas in an SEM graph (Figure 1A, upper panel of figure 1B) were chosen for analysis, and four elements, Al, O, Na and Cl, were analyzed. SEM/EDS showed the presence of all four elements analyzed (Figure 1B-C). The spectrum analysis and EDS map indicate a homogeneous distribution of the elements, implying that the vaccine was uniformly distributed in the thin-film freeze-dried powder.

(A)

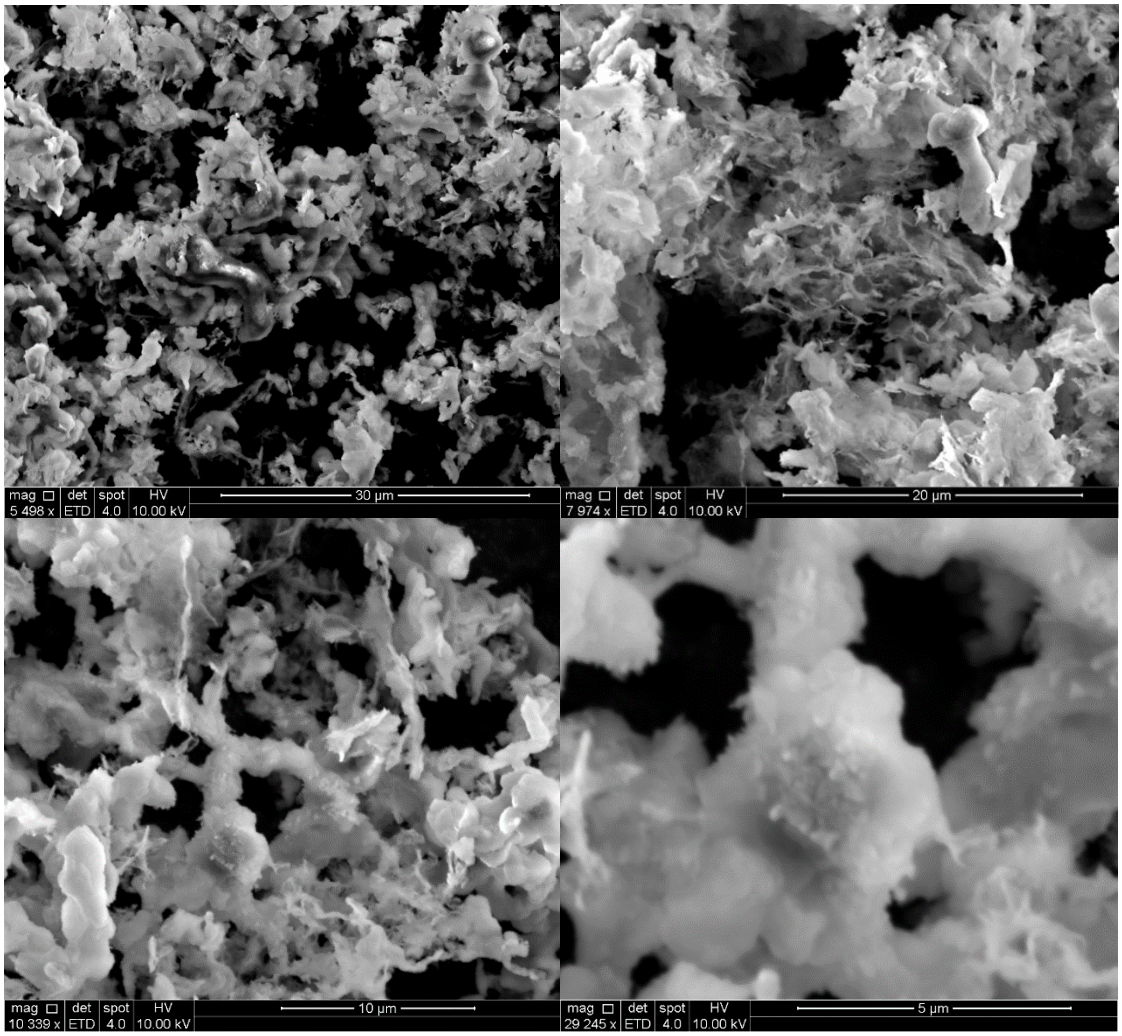


Figure 4.1 SEM/EDS of OVA-Alhydrogel® dry powder vaccine

(B)

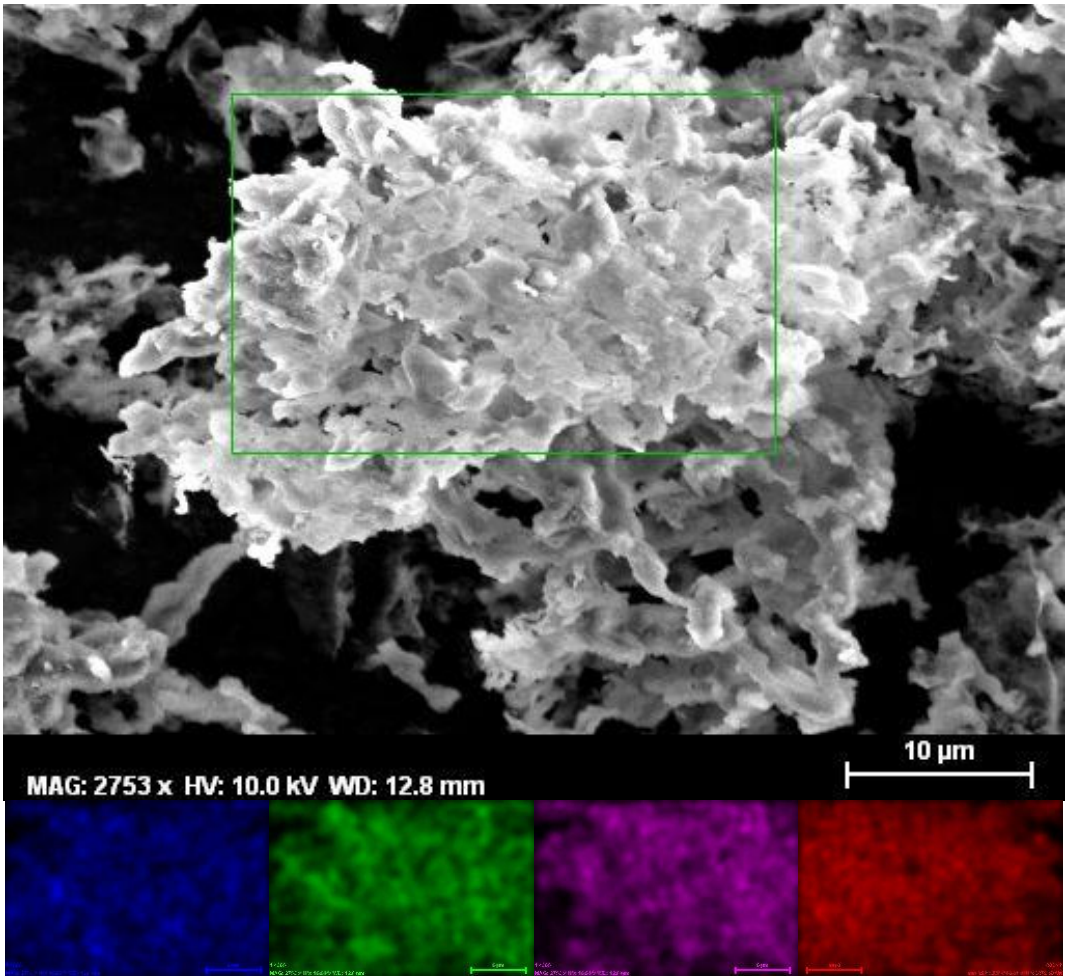


Figure 4.1 (Continued)

(C)

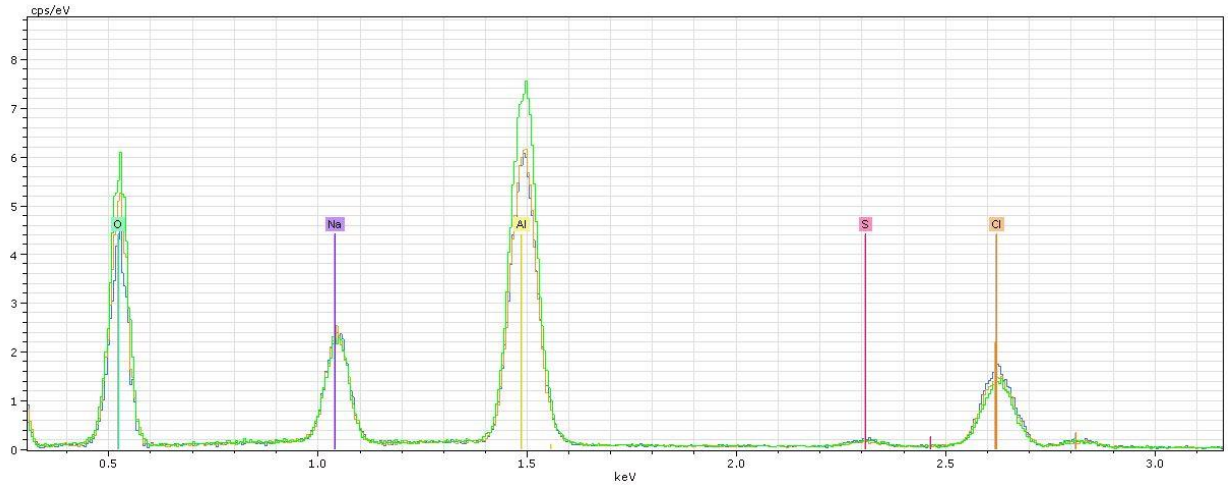


Figure 4.1 (Continued)

Shown in **(A)** are representative SEM images of the dry powder vaccine at different magnifications. **(B)** Randomly selected area in a SEM graph (upper panel), and representative elemental mapping (bottom panel). **(C)** EDS spectra of the elements tested (Al, O, Na, Cl; n = 3 random areas).

4.3.3 Intranasal dry powder delivery device and powder characterization

A new nasal dry powder delivery device was developed in our laboratory for this study. The device includes a housing reservoir and a pressurizing mechanism operable to pressurize gas/air chamber to desired pressure. The dry powder vaccine is loaded into the housing reservoir. Depressing the syringe plunger pushes air through the device, creating a powder plume that exits the orifice of the device (Figure 2A). The particle size of the OVA-Alhydrogel® dry powder vaccine was measured using a Malvern's Spraytec instrument and shown in Figure 2B. The median diameter of the vaccine dry powder was 12.55 ± 4.69 μm. According to FDA, particles greater than 10 μm are expected to deposit in the nasal cavities after nasal administration (194).

(A) **(B)**

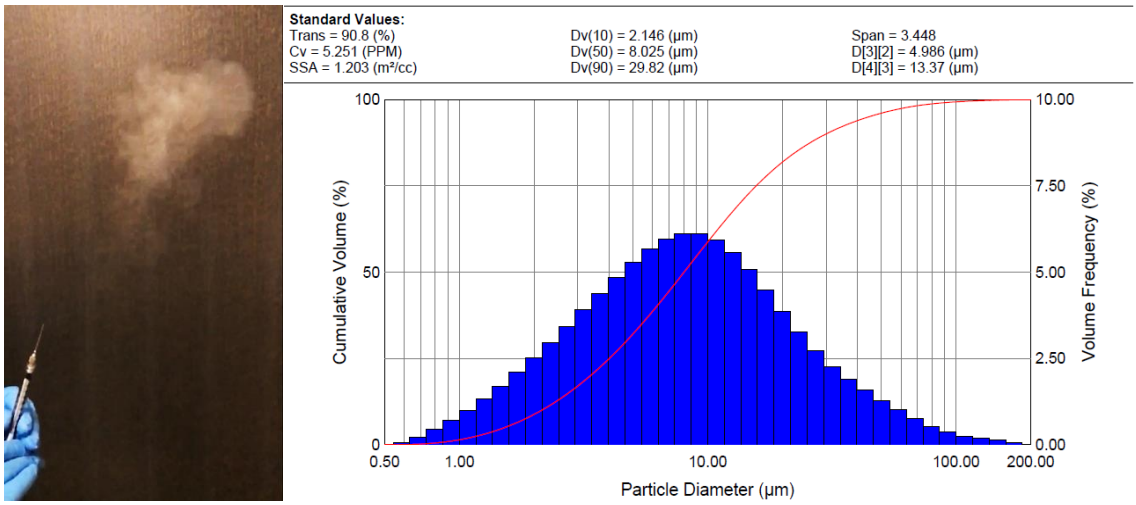


Figure 4.2 A powder cloud evolving from the dry powder delivery device and particle size distribution of the dry powder vaccine

(A) OVA-Alhyrogel® powder cloud evolving from the nasal dry powder delivery device. **(B)** A representative particle size distribution curves of the OVA-Alhydrogel® dry powder vaccine determined by Malvern Spraytec®.

4.3.4 Aerodynamic assessment of dry powder vaccine

Nose/nasal cavity is the easiest accessible part of the respiratory system. It is worth noting that release of antigen from the powder vaccine to the nasal cavity must take into account several factors, including wettability, dissolution rate, and the interaction of antigen-adjuvant with the mucus. However, for a nasal vaccine to afford protection, vaccines must present antigen to the target lymphoid tissues in nose. Nose-associated lymphoid tissue (NALT) in rodents refers to a pair of aggregated lymphoid tissues in the bottom of nasal ducts. In human nose cavity, the Waldeyer's ring, a well-known group of tonsils that includes the adenoid, tubal, palatine, and lingual tonsils, is the key lymphoid tissue, however (195). A post mortem study by Debertin et al. (2003) provided the first evidence of the existence of NALT, in addition to the Walderyer's ring, in young children (196). This study in young children found disseminated aggregates of lymphoid tissues in 38% of the cases, mainly found in the superior nasal meatus (30.1%), the middle concha (26.4%), the inferior nasal concha (13.5%), and the superior nasal concha (10.4%). Pabst stated that there is not any reported data on the frequency of NALT in adolescents and adults (197).

Evaluation of nasal deposition of the dry powder vaccine would be beneficial in the formulation development of nasal vaccines because of the fact that there are no guidelines or international consensus regarding the relationship between aerosol characteristics and deposition sites within the nasal cavities (198). We used nasal casts obtained from the CT scans of five adult humans to predict the deposition of the dry powder vaccine after nasal administration. Figure 3A shows the representative image of the different sections of the

nasal casts used. The deposition study is carried out at 15 L/min. As depicted in Figure 3B, 62.20 ± 8.14% the vaccine dry powder was recovered from the casts. Of the recovered powder, 64% was in the posterior nasal cavity. Overall, the nasal deposition can be deemed good, considering out of the total powder recovered (62.20 ± 8.14%), about 90% stayed in the nose, and only around 10% of the powder going to the post nasal fraction.

(A) **(B)**

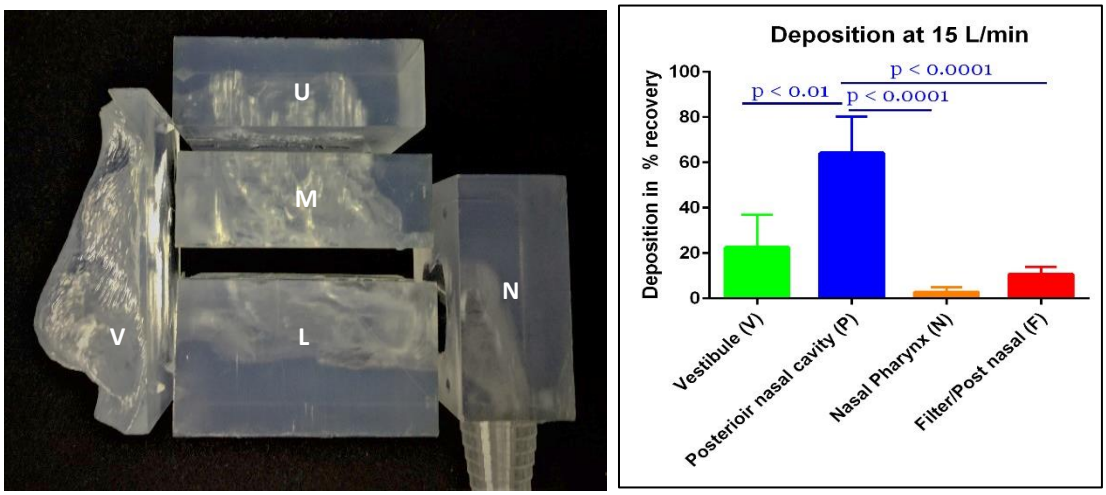


Figure 4.3 Deposition of the dry powder vaccine into a nasal cast

(A) A photo of the different sections of the nasal cast used. A - Anterior region making up the vestibule (V) and nasal valve area; U, M, L - upper, middle and lower turbinate regions, collectively called posterior nasal cavity (P); N - nasopharynx; and F - post-nasal fraction. **(B)** Deposition of the OVA-Alhydrogel® dry powder vaccine in nasal casts operated at 15 L/min. Data are mean ± S.D. from 5 adult casts. *** $p < 0.0001$, posterior nasal cavity (P) vs. Nasopharynx (N), *** $p < 0.0001$, posterior nasal cavity (P) vs. Filter/post nasal (F), and * $p < 0.01$, posterior nasal cavity (P) vs. vestibule (V).

4.3.5 The immunogenicity of the dry powder vaccine after intranasal administration

To evaluate the feasibility of administering the OVA-Alhydrogel® dry powder vaccine directly to the nose (IN Powder) to induce immune response, rats were nasally administered with the dry powder vaccine using our nasal dry powder delivery device. The immune responses induced by the same OVA-Alhydrogel® liquid vaccine after intranasal (IN Liquid) or subcutaneous (SC Liquid) administration were also assessed. Shown in Figure 4 are the OVA-specific antibody levels induced, i.e. serum IgG titers and mucosal IgA titers in nasal wash and BAL. Intranasal administration of the OVA-Alhydrogel® dry powder vaccine induced OVA-specific IgG response in rat serum samples in a level comparable to that induced by intranasal or subcutaneous administration of OVA-Alhydrogel® liquid vaccine (Figure 4A). Importantly, intranasal administration of the OVA-Alhydrogel® dry powder vaccine also induced OVA-specific IgA in rat nasal wash and BAL samples, whereas subcutaneous injection of OVA-Alhydrogel® liquid vaccine did not (Figure 4B-C). H&E staining of mouse nasal cavities did not reveal any difference among rats that received the OVA-Alhydrogel® dry powder intranasally, the OVA-Alhydrogel® liquid vaccine intranasally or subcutaneously, or normal saline intranasally (Figure 5), indicating that the OVA-Alhydrogel® dry powder vaccine was well tolerated locally when given intranasally.

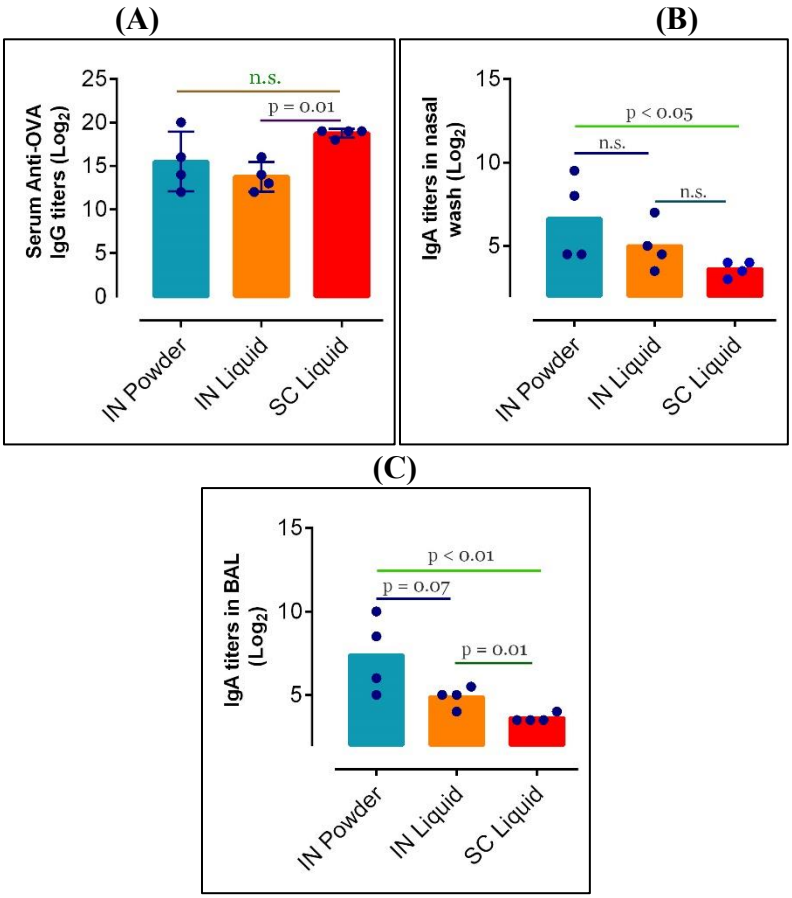


Figure 4.4 The immunogenicity of the dry powder vaccine after intranasal administration

Serum anti-OVA IgG titers and mucosal IgA titers in rats immunized with OVA-Alhydrogel® dry powder vaccine intranasally. Rats were dosed on days 0, 14 and 28 with the dry powder vaccine intranasally (IN Powder, n = 4), the liquid vaccine intranasally (IN Liquid, n = 4), or subcutaneously with the liquid vaccine (SC Liquid, n = 4). The dose of OVA was 20 µg per rat, and 400 µg for Alhydrogel®. The anti-OVA IgG titers in serum samples **(A)**, OVA-specific IgA titers in the nasal washes **(B)** and BAL samples (**C)** were measured 28 days after the third immunization. In **(A)**, ** p = 0.001, IN Liquid vs SC Liquid. In **(B)**, * p = 0.05, IN Powder vs. SC Liquid; in **(C)**, * p = 0.01, IN Powder vs. SC Liquid and * p = 0.01, IN Liquid vs SC Liquid.

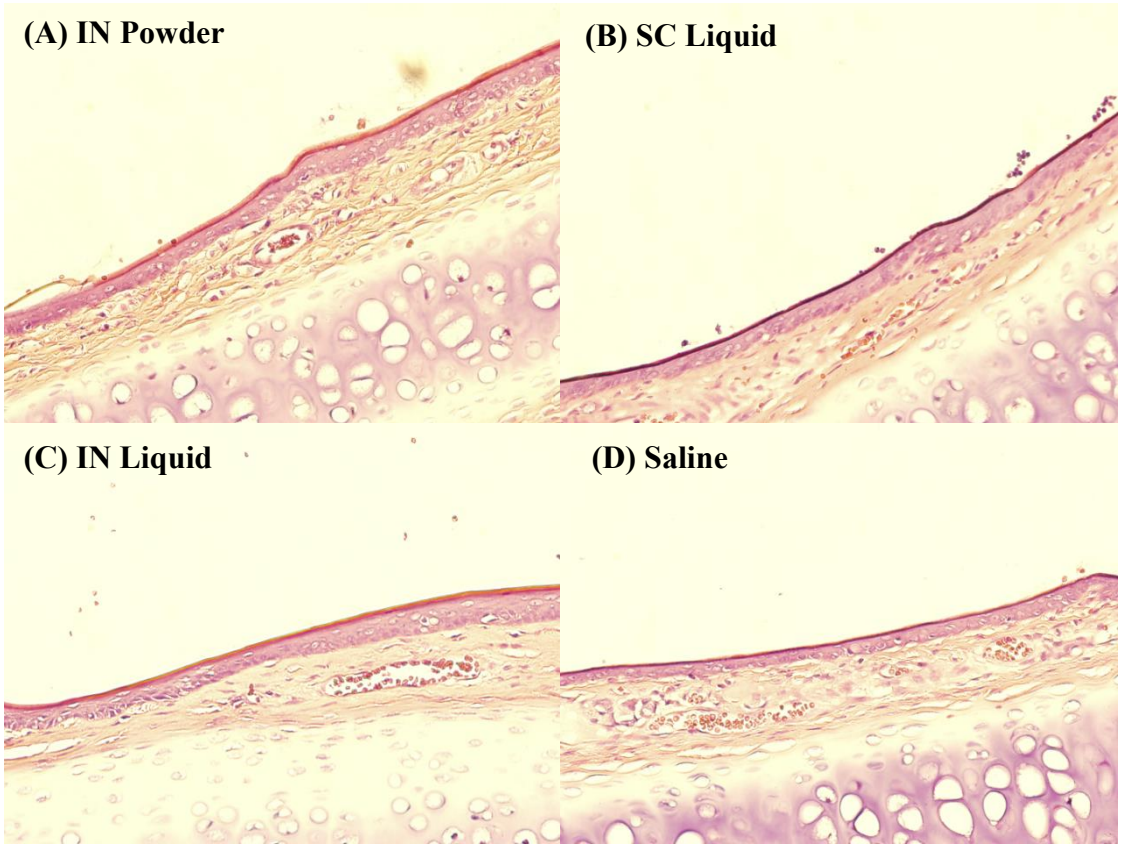


Figure 4.5 Histological examination of the nasal tissue after intranasal administration

Representative histological pictures of rat nasal epithelium. Shown are H&E stained images of the rats intranasally dosed with OVA/Alhydrogel® dry powder vaccine (IN Powder) **(A)**, s.c. dosed with OVA/Alhydrogel® liquid vaccine (SC Liquid)) **(B)**, intranasally dosed with OVA/Alhydrogel® liquid vaccine (IN Liquid) **(C)**, or intranasally dosed with normal saline (Saline) **(D)**.

4.3.6 Aluminum biodistribution in brain

Aluminum containing adjuvants possess excellent safety profile of close to a century (12, 29, 199), and it was suggested that all the injected aluminum hydroxide may be dissolved and absorbed eventually (200, 201). Due to the large size of the particulates in the OVA-Alhydrogel® vaccine dry powder and the OVA-Alhydrogel® liquid vaccine (i.e. X_{50} of ~12 μm and 8 μm, respectively), the OVA-Alhydrogel® particles are expected to largely stay in the nasal cavity after nasal administration (202, 203). However, there is a potential of brain exposure of aluminum via the olfactory epithelium (204-206). In some studies, no significant brain aluminum levels were seen in rats after 4 weeks of continuous exposure of insoluble aluminum (oxy)hydroxide (205), while others showed elevated brain levels of rabbits after one month of continuous nasal exposure of soluble aluminum in solutions (e.g. aluminum lactate or aluminum chloride, as 'Gelfoam') (206).

Therefore, we measured aluminum levels in the brain tissues of the immunized rats after terminal euthanasia (i.e. four weeks after the last immunization). Figure 6 shows the levels of aluminum determined in rat brain tissues. There was not any significant difference in aluminum levels among all four groups. This indicates that intranasal administration of the OVA-Alhydrogel® vaccine, in liquid or dry powder form, will not likely cause more toxicity in the brain than subcutaneous injection of the same OVA-Alhydrogel® liquid vaccine. For rodents, about 50% of the nasal cavity is lined with olfactory epithelium, compared to 3% for humans (207), which will likely further limit nose to brain transport in humans, if any. Therefore, it is very unlikely that transient nasal exposure of the

insoluble aluminum salt in vaccines for 1-3 times at a relatively low dose would result in an elevated level of aluminum in brain.

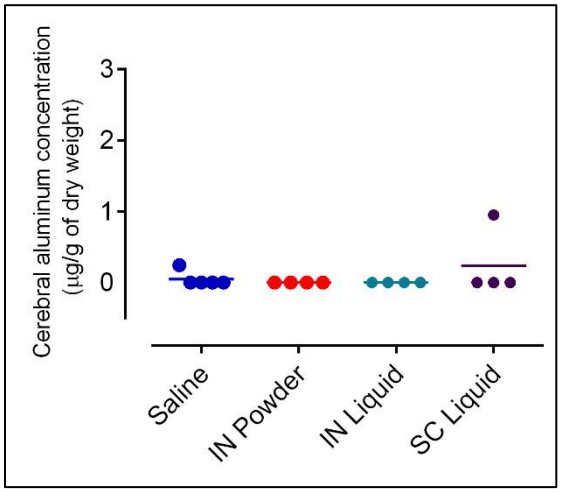


Figure 4.6 Aluminum biodistribution in brain after intranasal administration

Aluminum levels determined in rat brain tissues (µg/g of dry weight). Brain was collected 28 days after the third immunization, desiccated, and then incinerated before determining aluminum content in the samples using ICP-MS.

As mentioned earlier, dry powder vaccines have the potential to overcome the stability issue of liquid vaccines (i.e. sensitive to freezing and heat). There have been a few reported studies investigating intranasal delivery of dry powder vaccines (184-187). However, to our best knowledge, the present paper represents the first study testing the feasibility of intranasal immunization with an aluminum salt-adjuvanted dry powder vaccine. Existing human nasal vaccines (e.g. FluMist) are live attenuated vaccines (208). Subunit vaccines provide more challenges for nasal immunization, in part due to the low immunogenicity of subunit protein antigens, as compared to live attenuated vaccines (209). Therefore, the availability of proper adjuvant(s) plays a critical role for successful nasal mucosal immunization. For decades, scientists have been searching for a safe and effective nasal mucosal vaccine adjuvant, with little success. Aluminum salts in existing injectable human vaccines may be repurposed as nasal mucosal vaccine adjuvants. Furthermore, administering aluminum salt-adjuvanted vaccine powder intranasally can potentially address the cold-chain requirement associated with aluminum salt-adjuvanted liquid vaccines as well as the limitations associated with hypodermic needle-based injection.

4.4 CONCLUSION

In conclusion, in the present study we presented data demonstrating the feasibility of intranasally administering aluminum salt-adjuvanted dry powder vaccine to induce specific mucosal and systemic immune responses in a rat model. New and existing aluminum salt-containing vaccines may be converted into dry powder using our thin-film freeze-drying technology and administered without the need for needles using our nasal dry powder delivery device.

Chapter Five

Conclusion and perspectives

5.1 FUTURE OF INTRANASAL VACCINES:

The intranasal dry powder vaccines provide an opportunity to remove needles from traditional immunization programs, improve vaccine efficiency, and significantly impact healthcare in developing nations. The popularity of FluMist^{TM} indicates that intranasal dry powder vaccines has long time to stay (210). Cost of immunizations is an important factor in accepting new methods. It is possible that intranasal dry powder vaccinations might push the costs of immunizations higher. The primary reason is developing these vaccine formulations using sophisticated technologies to manufacture in a way to maintain highest level of activity and then developing world-class devices to deliver the vaccines to proper regions in the nose. Nevertheless, all methods have limitations and each method will find its niche application.

Despite recent developments, the overall picture is one of cautious optimism, enthusiasm, energy, and dedication. Vaccine development is in a dynamic phase and more people are being reached with vaccines. There are some barriers on the way. In past decade, there has been significant advancement has been done to overcome the issues with vaccine stability and also in the field of intranasal pharmaceutics. To capitalize on the intranasal dry vaccine powders, it is necessary to resolve the issues surrounding the development process. However, recently MannKind corporation and TechnoVax Inc have combined their technologies to create a powder formulation of VLP Influenza vaccine for

intrapulmonary self-delivery by inhalation. This 'inhaled vaccine powder' for influenza is shelf-stable and self-inhaled (211), gives us an indication that we are not too far from having first intranasal dry vaccine powder in market.

APPENDIX

Aluminum salt nanoparticles induce uric acid production[3]

A.1 INTRODUCTION

Many human vaccines contain insoluble aluminum salts as adjuvants (29, 30). Although insoluble aluminum salts have been used in vaccines for decades, their exact mechanisms of action have baffled scientists for years (31, 32). Over the years, various theories have been proposed to explain the mechanisms underlying the adjuvant activity of insoluble aluminum salts. It is clear now that aluminum salts work by a mixture of different mechanisms. Proposed mechanisms of immunopotentiation by aluminum-containing adjuvants include formation of antigen depot (33, 34), stimulation of antigen-presenting cells such as dendritic cells (DCs) (35), complement activation (36), and stimulation of chemokine release (31, 36). Moreover, there are reports showing that aluminum salts activate intracellular pathogen pattern recognition receptor signaling pathway involving the NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome (37, 38). Potent inflammatory cytokines such as IL-1β and IL-18 are released in response to NLRP3 activation, which direct the host responses to infection and injury (39). Furthermore, Kool and colleagues reported that the immunopotentiating effect of aluminum salts depends on the induction of uric acid (40).

[3]This chapter is based on "**Sachin G. Thakkar**, Haiyue Xu, Xu Li, Zhengrong Cui, ***Journal of Drug Targeting***" (Under review)

Previously we and others discovered that adjuvant activity of aluminum (oxy)hydroxide and aluminum (hydroxy)phosphate could be significantly improved by reducing the size of the particles in the aqueous suspensions of the insoluble aluminum salts from micrometer scale to nanometer scale (e.g. from 1-20 μm to ~100 nm) (41-44). We further provided evidence that the more potent adjuvant activity of the aluminum salt nanoparticles is likely related to their stronger ability in activating the NLRP3 inflammasome than aluminum salt microparticles (42).

In an effort to further elucidate the mechanisms underlying the stronger adjuvant activity of the aluminum salt nanoparticles, relative to microparticles, we evaluated and compared aluminum (oxy)hydroxide nanoparticles (AH-NPs) and microparticles (AH-MPs) in their ability to induce uric acid production by macrophages in culture and in a mouse model, hypothesizing that the AH-NPs are more potent than AH-MPs in inducing uric acid production.

A.2 MATERIALS AND METHODS

A.2.1 Reagents

Aluminum Hydroxide Nanopowder/Nanoparticles (high purity, 99.9%, # US3026) were from US Research Nanomaterials, Inc. (Houston TX). Polyvinylpyrrolidone, phosphate-buffered saline (PBS), ovalbumin (OVA), Sigma-Aldrich uric acid assay kit, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent were from Sigma-Aldrich (St. Louis, MO). Cell culture medium, penicillin, streptomycin, and fetal bovine serum (FBS) were from Invitrogen (Carlsbad, CA). Amplex® red uric acid assay kit was from Molecular Probes/Life Technologies (Eugene, OR). Mouse J774A.1 macrophage cells (# TIB-67™) were from the American Type Culture Collection (Manassas, VA).

A.2.2 Preparation and characterization of aluminum (oxy)hydroxide nanoparticles and microparticles

AH-NPs and AH-MPs were prepared as described previously (42). Briefly, the Aluminum Hydroxide Nanopowder was slowly added into warm water while stirring. The suspension was probe-sonicated and spun at 1000 rcf for 10 min. The supernatant was probe-sonicated repeatedly and spun down again at 1000 rcf for 10 min. The resultant supernatant in suspension was stabilized by adding polyvinylpyrrolidone (1%, w/v) and used as nanoparticles (AH-NPs). The sediment was re-suspended and used as microparticles (AH-NPs) in subsequent studies.

The AH-NPs and AH-MPs were examined using an FEI Tecnai Transmission Electron Microscope (TEM) available in the Institute for Cellular and Molecular Biology (ICMB) Microscopy and Imaging Facility at The University of Texas at Austin. Carbon-coated 400-mesh grids were activated for 1–2 min. One drop of the particle suspension was deposited on the grids and incubated for 2 min at room temperature. The grids were washed with water and air-dried for 1 min. Extra water was removed using filter paper and allowed to air-dry for 15 min before observation (41). The size of the particles was estimated based on randomly selected particles from the TEM micrographs. The aluminum contents in the AH-NPs and AH-MPs preparations were determined using a Varian 710-ES Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES) in the Civil Architectural and Environmental Engineering Department at The University of Texas at Austin. XRD analysis of the AH-NPs and AH-MPs in powder was performed using an R-Axis Spider with a Cu sealed tube source with a large, image plate detector (Rigaku, The Woodlands, TX) in the Chemical Engineering Department at The University of Texas at Austin. The particles were lyophilized into powder before XRD analysis. All XRD patterns were collected with a step size of 0.01 and counting time of 1 s per step over a 2θ range of 10 to 80.

A.2.3 MTT assay and quantification of uric acid in cell culture medium

Mouse J774A.1 macrophage cells were grown in DMEM supplemented with 10% FBS (v/v), 100 U/mL of penicillin and 100 μg/mL of streptomycin. MTT assay was used to determine cell viability after J774A.1 cells (2500 cells/well) were cultured with AH-NPs

or AH-MPs (aluminum content, 173 μg/well) for 72 h. Briefly, 20 μL of MTT reagent (5 mg/mL) was added to the wells and incubated at 37° C in the dark for 3-4 h. Two hundred microliters (200 μL) of dimethyl sulfoxide was added into each well and incubated for an additional 15 min to solubilize the MTT-formazan product. Absorbance was measured at 570 nm. A cell viability of greater than 90% was considered non-toxic.

To determine uric acid production, J774A.1 cells were incubated with the AH-NPs or AH-MPs as mentioned above, and uric acid concentration in the cell culture medium was determined using a Sigma-Aldrich uric acid assay kit following the manufacturer's instructions.

A.2.4 Animal study

The animal study was conducted following the U.S. National Research Council guidelines for care and use of laboratory animals. Animal protocol was approved by the Institutional Animal Care and Use Committee at The University of Texas at Austin. Female BALB/c mice (18–20 g, Charles River Laboratories, Wilmington, MA) were injected intraperitoneally (i.p.) with OVA-adsorbed AH-NPs (6 mice) or OVA-adsorbed AH-MPs (5 mice), both in a 0.9% sterile NaCl solution. The dose of aluminum was 263 μg per mouse, and the dose of OVA was 10 μg per mouse. As controls, mice were injected with a 0.9% NaCl (5 mice) solution or OVA adsorbed on Alhydrogel® (aluminum, 263 μg; OVA, 10 μg). Peritoneal lavage was collected 6 h later. Uric acid levels in the lavage samples were determined using the Amplex® red uric acid assay kit following the manufacturer's instructions.

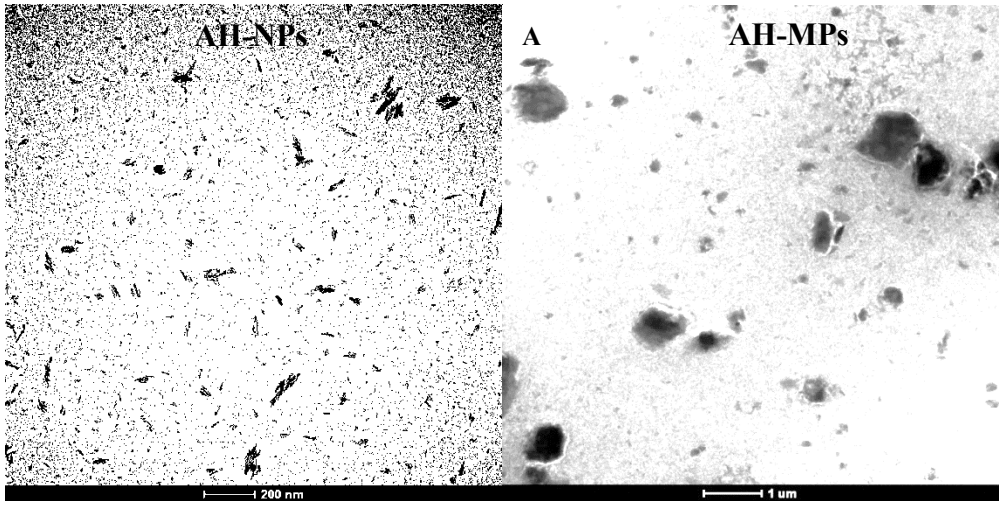
A.2.5 Statistical analysis

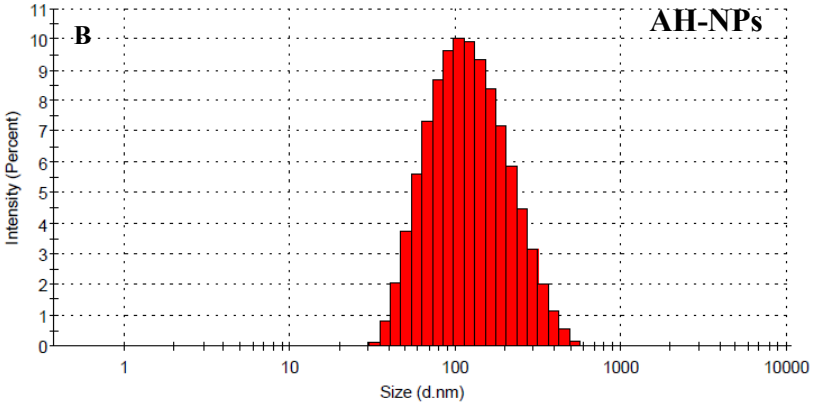
Statistical analyses were completed by performing two-tailed Student's t-test for two-group analysis or one-way ANOVA followed by Tukey's post hoc analysis for multiple group comparisons (GraphPad Prism 7 software, La Jolla, CA). A p value of $\leq$ 0.05 (two-tail) was considered significant.

A.3 RESULTS AND DISCUSSION

Previously, we and others showed that the adjuvant activity of aluminum salt nanoparticles is significantly stronger than the microparticles (41-44). It was also reported by Kool and colleagues that the immunopotentiating effect of Imject^{TM} Alum, a preparation that contains aluminum hydroxide and magnesium hydroxide, depends on stimulating uric acid release *in vivo.* They have found very high levels of the endogenous danger signal uric acid in the peritoneal lavage of mice after they were i.p. injected with OVA-adsorbed Imject^{TM} Alum (1 mg aluminim hydroxide per mouse). In addition, degradation of uric acid by pretreating mice with uricase abolished OVA-specific $CD4^+$ T cell priming, although the effect of the uricase pretreatment on specific antibody response was not reported (40). Therefore, we hypothesize that the stronger adjuvant activity of aluminum (oxy)hydroxide nanoparticles (AH-NPs), relative to aluminum (oxy)hydroxide microparticles (AH-MPs), is related to difference in their ability to induce uric acid production, and the present study was designed to test that hypothesis.

AH-MPs and AH-NPs were prepared from same aluminum (oxy)hydroxide aqueous suspension by centrifugation as described previously (42). Representative TEM images of the AH-NPs and AH-MPs are shown in Fig. 1A. Shown in Fig. 1B are representative particle size distribution curves of the AH-NPs and AH-MPs. The majority of the AH-NPs were below 100 nm in diameter, whereas the median diameter of the AH-MPs (i.e., X_{50}) was 9.43 μm, with X_{10} and X_{90} values of 1.69 μm and 17.74 μm, respectively (Fig. 1B). XRD analysis showed that the AH-NPs and AH-MPs have identical characteristic peaks, indicating that they have similar composition and





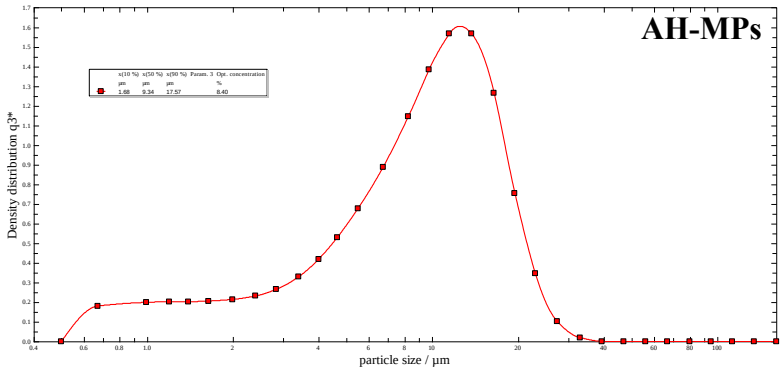


Figure A.1 Physicochemical characterization of aluminum (oxy)hydroxide nanoparticles and microparticles

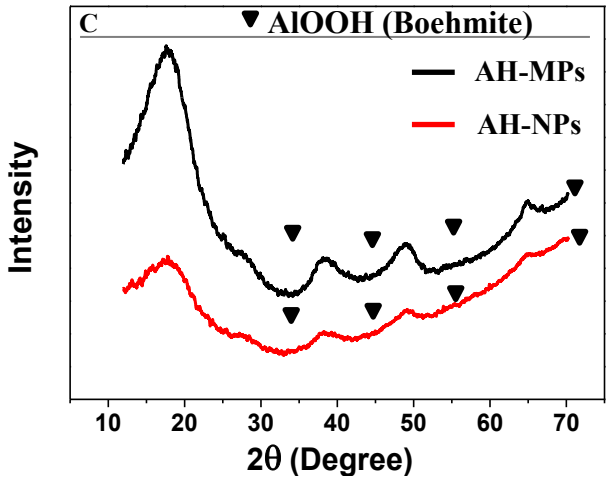


Figure A.1 (Continued)

(A) Representative TEM images of AH-NPs and AH-MPs. **(B)** Representative particle size and size distribution profiles of AH-NPs and AH-MPs as determined using dynamic light scattering and laser diffraction, respectively. **(C)** XRD patterns of the AH-MPs and AH-NPs.

crystallinity (Fig. 1C). Characteristic peaks of boehmite (aluminum oxyhydroxide (AlOOH)), based on the XRD spectra of the Joint Committee on Powder Diffraction Standards (PDF No. 00-021-1307), are labeled for both samples. The particles were mainly amorphous due to the huge characteristic peak in the 2θ range of 10-25, but they also contain some crystalline boehmite (Figure 1C) (44).

To test whether the AH-NPs and AH-MPs are different in inducing uric acid production in cell culture, J774A.1 mouse macrophages were incubated with AH-NPs or AH-MPs for 72 h, and uric acid levels in cell culture medium were measured. As shown in Fig 2A, AH-NPs induced uric acid production, whereas AH-MPs did not. Fig. 2B shows the cytotoxicities of AH-NPs and AH-MPs to J774A.1 cells after 72 h of incubation at the same concentration as in Fig. 2A. It appears that the AH-NPs induced cell death, whereas the AH-MPs at the same aluminum concentration did not cause any significant cell death (i.e., 71% vs. 100% survival, $p < 0.0001$). Cell death or damage causes the release of uric acid, explaining why incubation of J774A.1 cells with AH-NPs caused the release of uric acid in cell culture medium, but incubation of same number of cells with the AH-MPs at the same aluminum concentration did not (Fig. 2A).

To evaluate and compare the AH-NPs and AH-MPs on their abilities to induce uric acid production *in vivo*, AH-NPs and AH-MPs were surface-adsorbed with OVA as a model antigen because for immunization, aluminum salts are not injected without an antigen *in vivo*. Kool and colleagues also injected mice with Imject^{TM} Alum adsorbed with OVA in their study leading to the discovery of aluminum salt adjuvant's ability to induce uric acid production in mice (40). As shown in Fig. 2C, i.p. injection of BALB/c

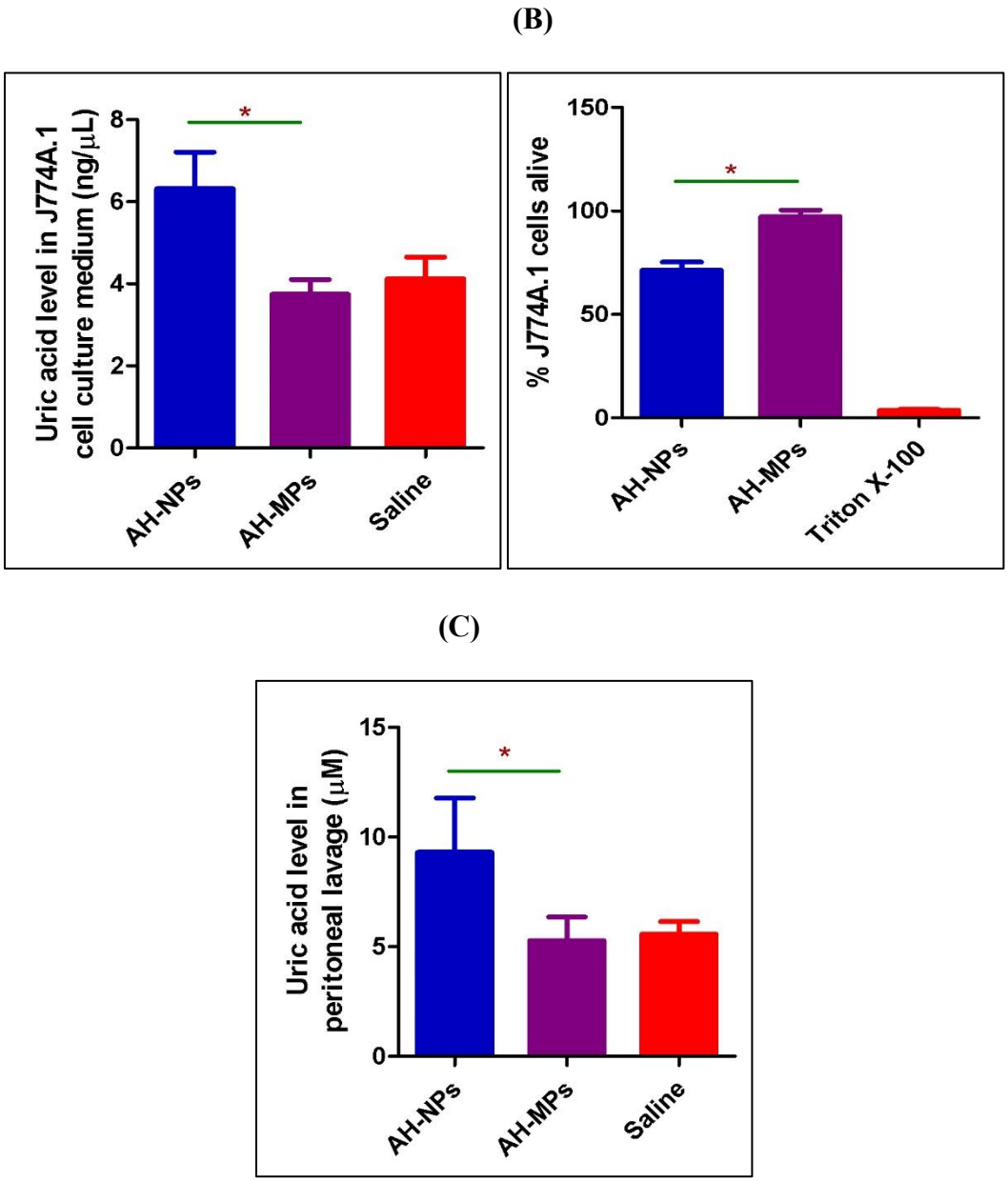


Figure A.2 Cytotoxicity and induction of uric acid by AH-NPs and AH-MPs

Figure A.2 (Continued)

(A) J774A.1 cells were incubated with AH-NPs or AH-MPs for 72 h, and cell viability was measured. Triton-X-100 was used as a positive control (* $p < 0.0001$, vs. AH-MPs). **(B)** J774A.1 cells were incubated with AH-NPs or AH-MPs for 72 h, uric acid levels in culture medium were determined. Data shown are mean ± SEM ($n = 5 – 6$). *, $p < 0.001$ vs AH-MPs. In A-B, the number of J774A.1 cells were 2500 cells/well, and the aluminum concentration was 173 μg/well. **(C)** Stimulation of uric acid production in BALB/c mice by AH-NPs and AH-MPs. Mice were i.p. injected with AH-NPs, AH-MPs, or normal saline, and after 6 h, uric acid levels were measured in the peritoneal lavage. AH-NPs and AH-MPs were adsorbed onto OVA (Aluminum, 263 μg/mouse; OVA 10 μg/mouse). Data shown are mean ± SEM ($n = 5 – 6$). *, $p < 0.005$ vs AH-MPs.

mice with OVA-adsorbed AH-NPs increased uric acid level in the peritoneal lavage of mice, as compared to that in mice i.p. injected with normal saline. However, i.p. injection of OVA-adsorbed AH-MPs did not increase uric acid production as compared to i.p. injection of normal saline (Fig. 2C), indicating that the potent vaccine adjuvant activity of the AH-NPs may be due, at least in part, to their stronger ability to induce uric acid production. It was unexpected, however, that our AH-MPs at the concentration or dose tested did not cause significant uric acid release in cell culture and in the mouse model (Fig. 2A, C). Imject™ Alum, which contains mainly microparticles (X_{10}, 0.96 μm; X_{50}, 3.24 μm; and X_{90}, 11.64 μm, Fig. 3A), were reported to induce significant local uric acid production after OVA-adsorbed Imject™ Alum was i.p. injected into mice (40). Of course, the dose the Imject™ Alum in Kool et al's study was 1 mg per mouse (i.e. 1 mg aluminum hydroxide or 0.346 mg aluminum), whereas the dose of aluminum in the present study was 0.263 mg per mouse. Moreover, the AH-MPs (i.e., X_{10}, 1.69 μm; $X_{50,}$ 9.43 μm; and X_{90}, 17.74 μm, Fig. 1(A)) used in the present study were generally larger than the particles in the Imject™ Alum (e.g. ~3-fold larger in X_{50}). Differences in composition, particle size and size distribution, and dose between Imject™ Alum and our AH-MPs may be responsible for their ability, or lack of ability, to induce uric acid release *in vivo*.

In fact, we also tested Alhydrogel®'s ability to induce uric acid release *in vivo*. Alhydrogel® (i.e., X_{10}, 0.67 μm; $X_{50,}$ 1.67 μm; and X_{90}, 29.21 μm, Fig. 3B) at an aluminum dose identical to our AH-MPs, adsorbed with OVA, was also capable of inducing uric acid production in mice (Fig. 3C). Alhydrogel® and our AH-MPs are both

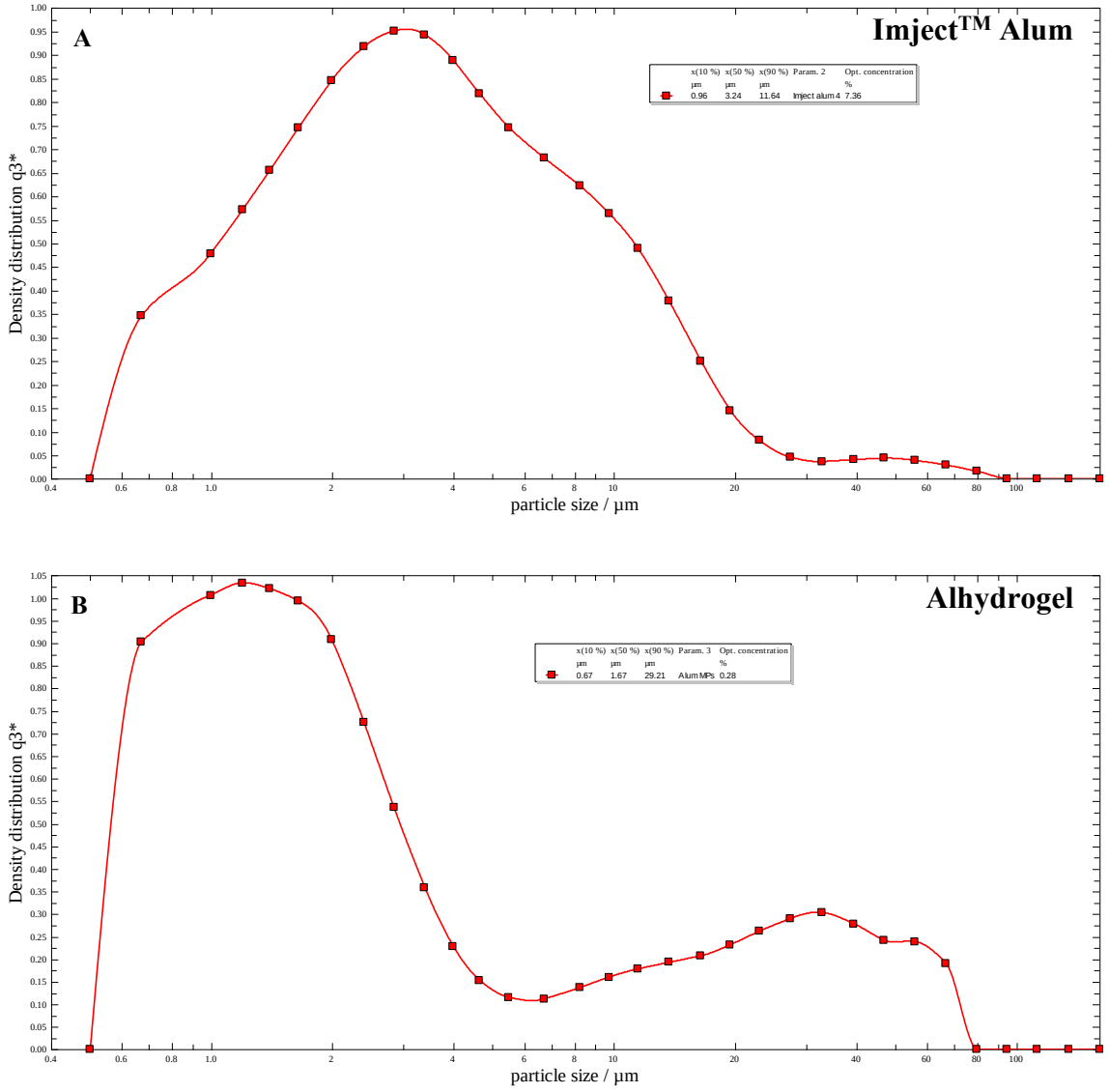


Figure A.3 Comparison of Alhydrogel and Imject with AH-NPs and AH-MPs

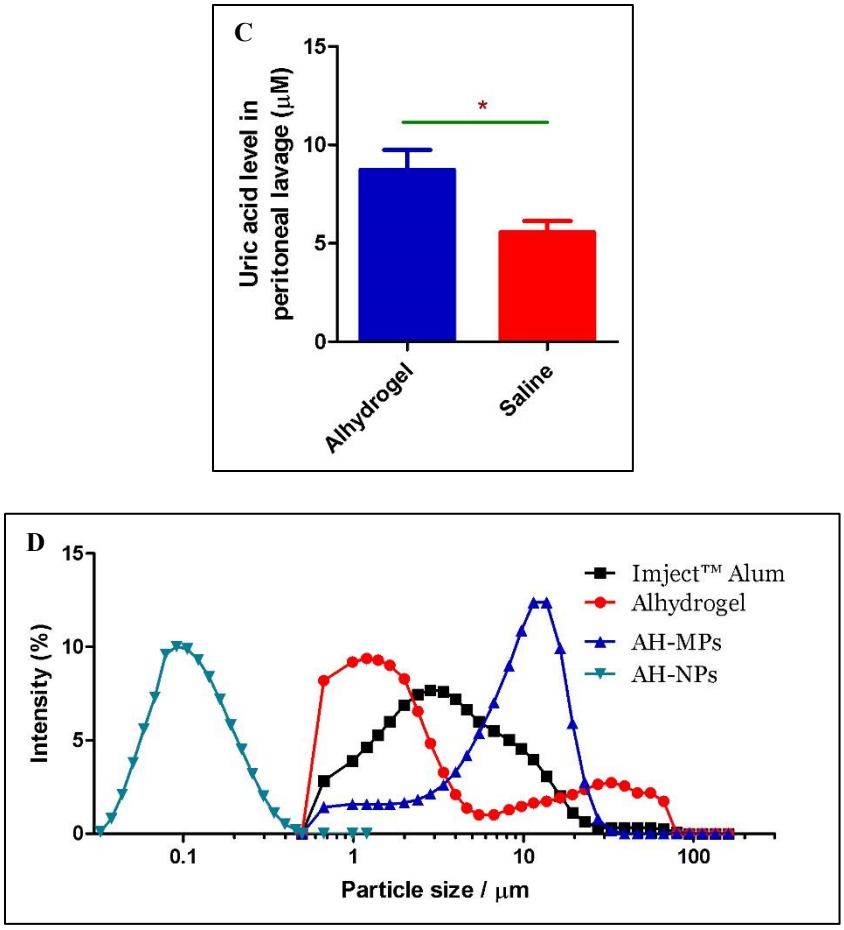


Figure A.3 (Continued)

(A-B) Representative particle size and size distribution profiles of Alhydrogel® **(A)** and Imject™ Alum **(B)**, respectively, as determined by laser diffraction. **(C)** Uric acid induction by Alhydrogel®. BALB/c mice were i.p. injected with OVA-adsorbed Alhydrogel (Aluminum, 263 μg/mouse; OVA 10 μg/mouse) or normal saline. Uric acid levels were determined in the peritoneal lavage after 6 h. Data shown are mean ± SEM (n = 5). *, $p < 0.0005$ vs. normal saline. **(D)** Overlay of particle size distribution curves of AH-NPs, AH-MPs, Imject™ Alum, and Alhydrogel®. The vertical dashed line indicates a hypothetical particle size value; aluminum salt particles smaller than that can induce uric acid release, whereas those larger than that cannot.

mainly aluminum oxyhydroxide in composition, although Alhydrogel® has moderately high crystallinity (44), whereas our AH-MPs are mainly amorphous (Fig. 1C) (44). Therefore, it is very likely that the particle size and size distribution of aluminum salts significantly affect their ability to induce uric acid production. It is also possible that the smaller aluminum salt particles in Alhydrogel® and Imject™ Alum, although not as small as the particles in our AH-NPs, are capable of inducing uric acid production, whereas those relatively larger aluminum salts, especially the very larger ones in our AH-MPs (i.e. X_{50} = 9.43 μm), are not. The upper limit of the size of the aluminum salt particles that are capable of inducing uric acid production remains unknown, but very large aluminum salt particles such as the ones with a diameter ≥ 10 μm may be too large for antigen-presenting cells such as mouse DCs and macrophages to internalize them, preventing them from causing cell damage or death and inducing uric acid release. The reported average size of the rat alveolar macrophages is around 13 μm (212-214). We have measured the size of the J774A.1 mouse macrophage cells under microscope and found it to be 16.90 ± 2.47 μm (n = 50 cells). There are reports of macrophage uptake of particles larger than themselves, but the majority of the aluminum (oxy)hydroxide particles in our formulation may be too large for efficient uptake by mouse DCs or macrophages. Flach et al. (2011) even reported that mouse DC2.4 cells and human DCs differentiated from THP-1 cells did not internalize aluminum salt particles of ~5 μm (215). Instead, those particles induced abortive phagocytosis (215). Therefore, it is not unreasonable to postulate that the mechanisms by which smaller aluminum salt particles and larger ones potentiate immune responses are different (e.g. smaller vs larger than 4 μm (Fig. 3D)). Uric acid release may be related to

the adjuvant activity of smaller aluminum salt particles, but not that of larger particles in traditional aluminum salt adjuvant such as Alhydrogel®. Of course, it is possible that our AH-MPs at higher concentrations may cause cell damage and could in turn induce uric acid release as well.

A.4 CONCLUSION

In conclusion, the potent adjuvant activity of the aluminum (oxy)hydroxide nanoparticles is likely related to their ability to induce uric acid release, as compared to large aluminum (oxy)hydroxide microparticles. In traditional insoluble aluminum salt-based vaccine adjuvants, the mechanisms by which relatively smaller particles potentiate immune responses may be different from relatively larger ones.

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